

Studies of endocrine abnormalities in relation
to various disturbances of electrolyte metabolism.

by

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Chapter I : Introduction

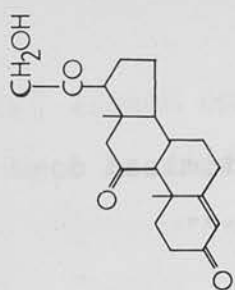
Introduction.

General.

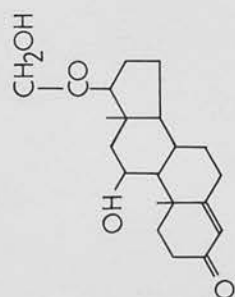
This thesis is concerned with the measurement of the endogenous hydrocortisone production rate by isotope dilution in healthy human subjects and its application a) to the diagnosis and treatment of patients with endocrine disorders and b) to the understanding of the role of the adrenal gland in the metabolic response to surgery. The availability of ^{14}C -labelled hydrocortisone with highly sensitive instruments to measure its activity and the use of chromatography and fluorimetry are the main contributing factors which render the methods used in this investigation more reliable than the existing conventional tests for adrenal function.

It is now well established that the adrenal cortex plays an important role in human physiology. The hormones it secretes into the blood affect carbohydrate and protein metabolism; electrolyte and water distribution and excretion; sex functions and characteristics; appetite, strength and general well being; and the reaction of the body to infection, cold, trauma and shock.

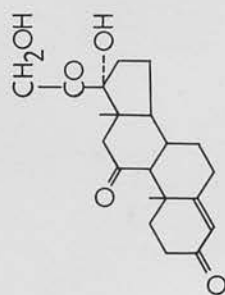
But it was Thomas Addison who in 1855, first suggested that the adrenals were essential to health and that death occurred in their absence when he recorded the symptoms resulting from the destruction of the adrenal glands - a description of the disease which now bears his name. Few were impressed by Addison's discovery at that time although it did prompt some experimental work in which the adrenals of animals were removed with very equivocal results.



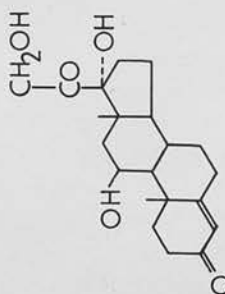
Dehydrocorticosterone
Kendall's A



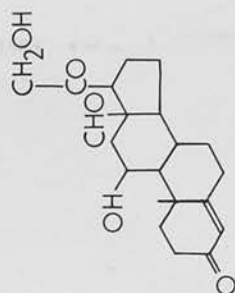
Corticosterone
Kendall's B



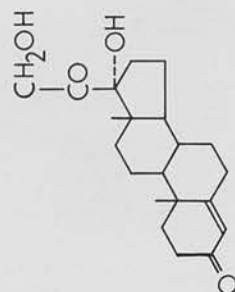
Cortisone
Kendall's E



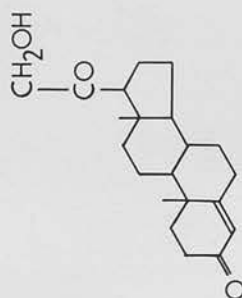
Hydrocortisone
Kendall's F



Aldosterone



17-OH Cortisone
Compound S. (Reichstein.)



Cortexone

Figure 1: Seven Biologically active Corticosteroids.

The next big move came in the beginning of the 20th Century when investigations carried out in several laboratories indicated the probability that the adrenal cortex possessed a hormone indispensable to life. Thus Stewart and Rogoff (1927) were able to prolong the life span of adrenalectomised dogs by administering to them the crude but physiologically active extract obtained from dogs' adrenals. Likewise Hartman et al (1927) also reported the prolongation of lives of adrenalectomised cats with the adrenocortical principle extracted from beef adrenals.

Attempts to isolate this active principle in its pure form and the determination of its chemical structure with a view to its synthesis soon followed. Work in this direction revealed the isolation not of one but several substances in the adrenocortical extract. During the period 1936-42, nearly 30 different steroids were isolated and since then some 10 or more have been added to the list (Neher 1958). But it was the pioneering work especially of Kendall, Wintersteiner and Reichstein that shed considerable light on the chemical structure and physiological significance of this large group of compounds isolated from the adrenal cortex.

The adrenal cortex, as is understood today, produces C₁₉ steroids (androgens), C₁₈ steroids (oestrogens) and C₂₁ steroids (corticosteroids). Most of the biologically active and inactive steroids are C₂₁ steroids and all of them have a highly oxidised side chain attached to C-17. Also, most of them have an oxygen atom at C-11 and Figure 1 shows seven of the biologically active corticosteroids. It may also be noted that they all possess a double bond between C-4 and C-5 atoms and an oxygen atom at C-3, an arrangement commonly referred to as a Δ^4 -3-ketone group./

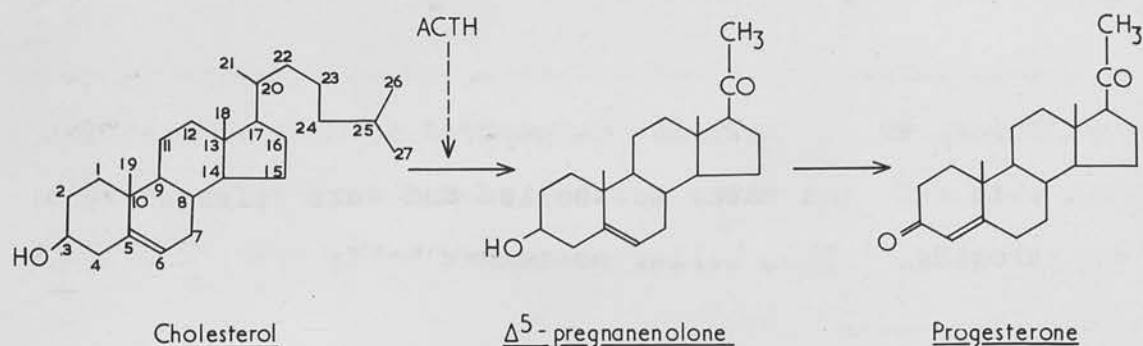


Figure 2: Biosynthesis of Progesterone.

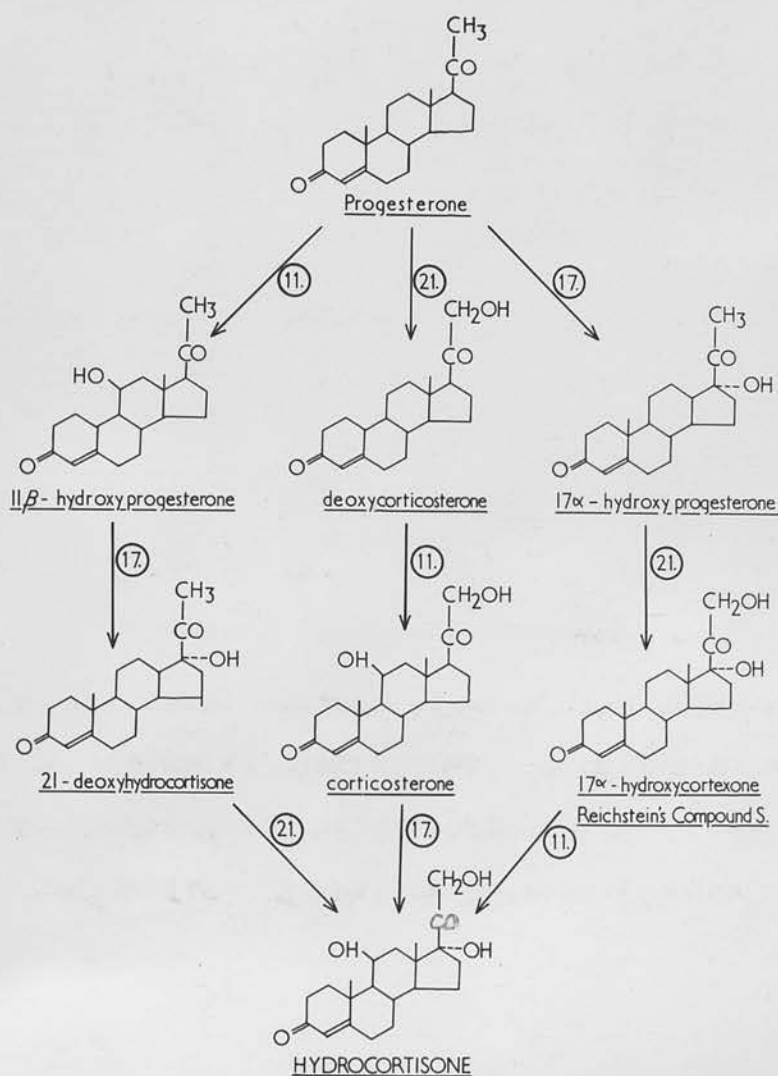


Figure 3: Anabolism of Hydrocortisone.

group. Before the discovery of aldosterone, it was generally believed that corticosteroids with an oxygen atom at C-11 were concerned with carbohydrate metabolism and were referred to as glucocorticoids, while those not oxygenated at C-11 were actively connected with salt and water metabolism and were referred to as mineralcorticoids. This belief no longer holds since aldosterone has substituents at C-11 and at C-18.

Metabolism.

The fundamental building block of androgens, oestrogens and corticosteroids is progesterone which is believed to be derived from cholesterol. Figure 2 briefly illustrates the biosynthesis of progesterone from cholesterol. As this thesis will be dealing with the measurement of production rate of hydrocortisone, only the anabolism and catabolism of hydrocortisone will be briefly reviewed.

In the adrenal cortex, progesterone is converted into hydrocortisone by successive hydroxylation at the 17, 21 and 11 positions. Figure 3 shows that the three metabolic routes, all lead to the synthesis of hydrocortisone. It is not certain at what stage the anterior pituitary adrenocorticotrophic hormone (ACTH) acts in accelerating corticosteroid biosynthesis, but it is believed at an early one, such as the conversion of cholesterol to pregnanenolone, see Fig.2. Intravenous infusions of ACTH in normal subjects produce a rise in blood hydrocortisone and in the urinary excretion of hydrocortisone metabolites. The elevated concentration of plasma hydrocortisone acts as a brake on ACTH production, thus establishing a self adjusting system.

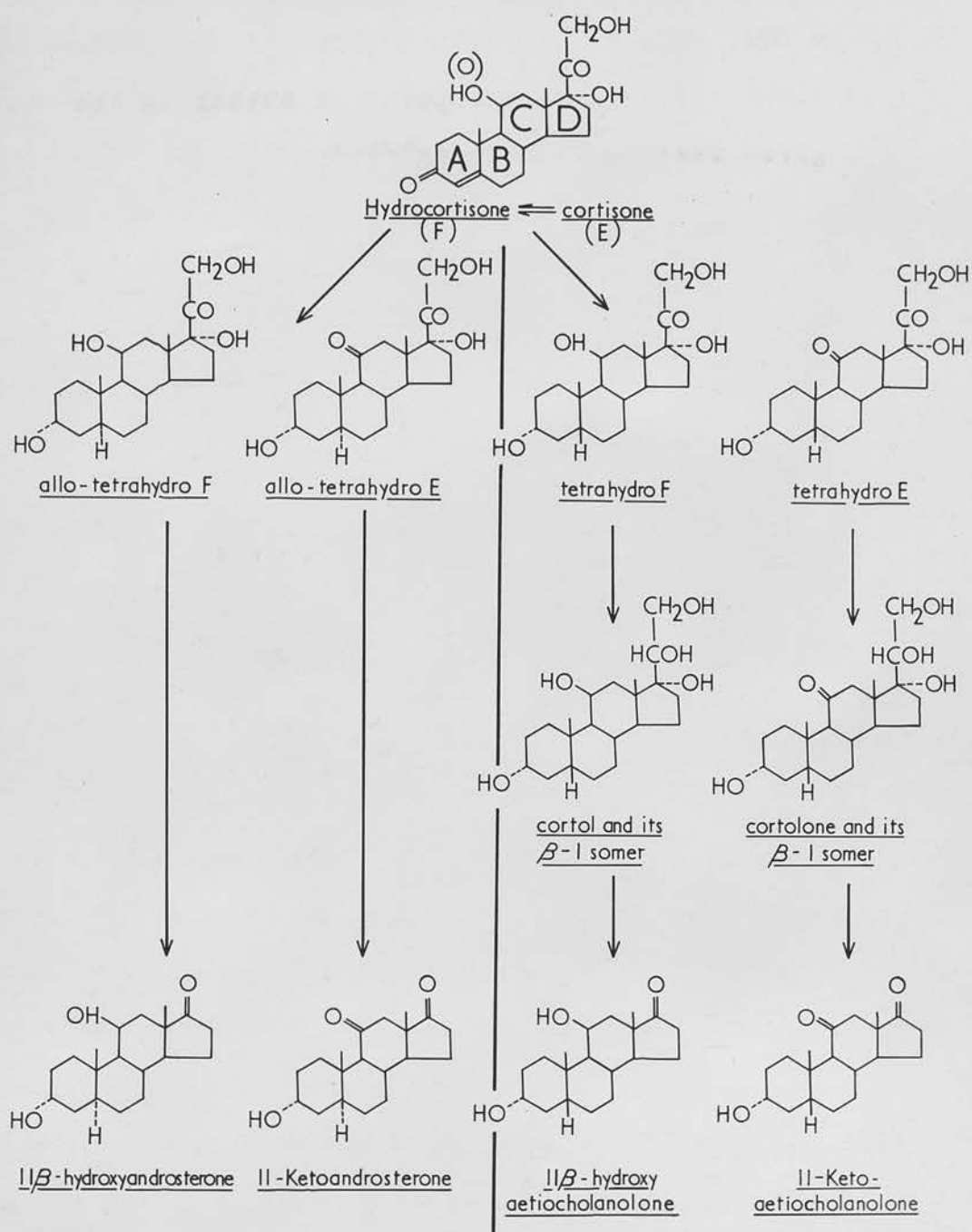


Figure 4: Catabolism of Hydrocortisone.

The principal pathways of hydrocortisone catabolism are shown in Figure 4. The first step involves the reduction of Ring A and conversion of the ketone at C-3 into a hydroxyl group. This step common to most adrenal steroids results in the production of tetrahydro derivatives. The next point of attack is the side chain; this gives rise to 20-hydroxylated compounds - cortol, cortolone; β -cortol, β -cortolone - (Fukushima et al 1955), and if the cleavage of the side chain is complete, to 11-oxygenated 17-ketosteroids. All these metabolites are conjugated in the liver mainly with glucuronic acid and sulphuric acid to form water soluble products which are then excreted in the urine; only a very small proportion of the excreted steroids or their metabolites are in the free state.

Methods of Adrenocortical Assessment

Biological Methods.

Because derangement in adrenocortical secretions causes various metabolic disturbances, methods for assessing adrenal activity have attracted widespread interest. The earliest attempts resorted to biological methods. The urine of the subject under investigation was extracted of its cortin activity and tests were carried out on adrenalectomised rodents. Assessment of activity depended upon the ability of the physiologically active steroids present in the extract to produce certain effects on the animal. One of the earliest was that of Seyle and Schenker (1938) who used the ability of the urinary extracts to prolong the survival time of adrenalectomised rats on exposure to low environmental temperature. In 1946, Venning et al had observed that the administration of /

of adrenal cortical extracts to adrenalectomised rats resulted in the deposition of liver glycogen in the fasting animal and these workers adapted this as a method for estimating the activity of the urine extract. In 1951, Speirs and Meyer described a method which showed that the extent of fall in the circulating eosinophils in the adrenalectomised mice on administration of the urine extract, could be related to the amount of adrenal steroids having an oxygen atom at C-11 present in the extract. Dorfman (1950), Simpson and Tait (1952) described similar methods for the estimation of mineral corticoids based on the retention of sodium and excretion of potassium in the adrenalectomised rat.

Although these biological assays provided valuable information regarding a particular type of response, they did not adequately reflect the quantitative adrenocortical secretory activity. Today, these have given way to chemical methods which are, by comparison rapid and simple enough for routine clinical purposes.

17-ketosteroids (17-KS)

The determination of urinary 17-ketosteroids was the earliest chemical method and from the clinical point of view, the most important to be used in diagnosis and treatment. The main steps in this estimation involve acid hydrolysis of the urine, extraction with organic solvents, washing of the extract with alkali to remove acidic material and the development of colour by application of the Zimmerman reaction which makes use of the reaction of the active methylene group at C-16 with *m*-dinitrobenzene in strong alkali.

Urinary 17-KS arise from precursors which have been secreted by the adrenal cortex, by the testes and possibly to some extent by the ovaries. In the male $\frac{2}{3}$ of the neutral 17-KS is of /

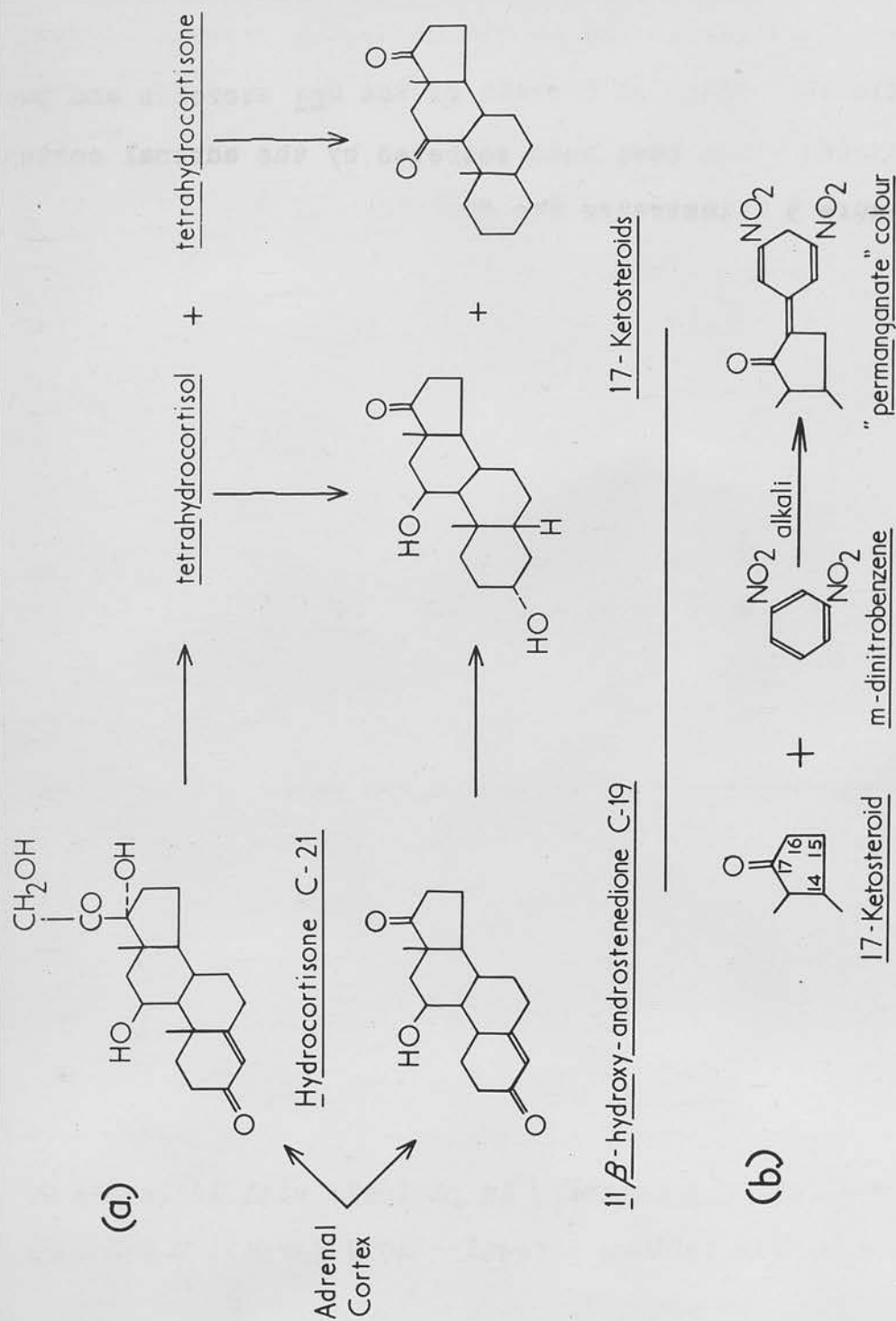


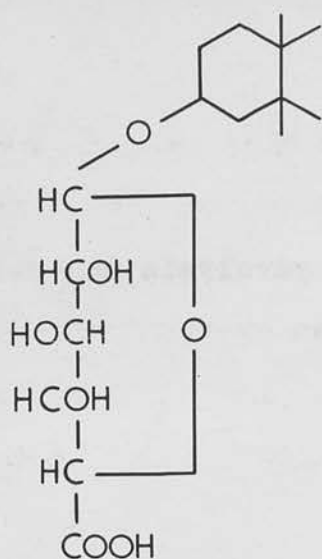
Figure 5: (a) Formation of 17-Ketosteroids and (b), the Zimmerman Reaction.

of adrenal origin and $\frac{1}{3}$ of testicular origin. In the female, practically all are elaborated by the adrenal cortex. The 17-KS of adrenal origin arise partly by the oxidative removal in the tissues of the side chain of certain of the C_{21} steroids and partly from C_{19} steroids which have been secreted by the adrenal cortex itself. Figure 5 illustrates the formation of 11-oxygenated 17-ketosteroids and the Zimmerman reaction.

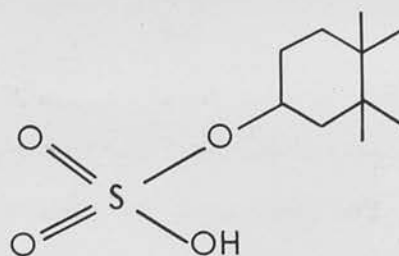
The total urinary 17-KS may be estimated either colorimetrically or polarographically but in the majority of cases the Callow-Zimmerman technique or some modification thereof is used. Recently a number of microtechniques have been proposed (Drekter et al 1947, 1952; Vestergaard 1951). In these procedures, only a few millilitres of urine are acid hydrolysed and extracted; the final extract being subjected to colorimetric analysis after application of the Zimmerman reaction.

Numerous workers have estimated the excretion of urinary 17-KS in normals and in a wide variety of clinical conditions. Investigations have shown that the output varies with age; it is low in young children and again declines in old age. In healthy adult males, the range is from 10 to 20 mg per day and in healthy adult females, from 5 to 15 mg in 24 hours. In patients with adrenocortical hyperfunction the level is generally increased if androgen production is affected; in patients with Addison's disease or anterior pituitary failure affecting ACTH formation and release, the output of 17-KS is reduced.

The 17-KS are excreted in the urine in the conjugated form rather than the free form. The actual nature of the conjugation is not yet known but there is ground to believe that the majority/

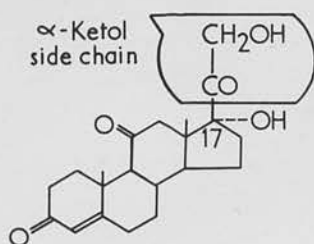


Glucuronide

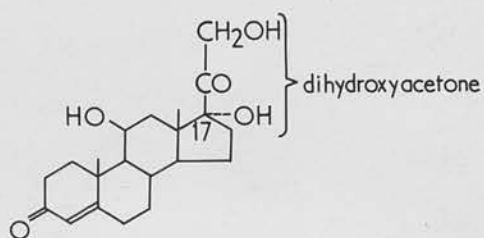


Sulphate

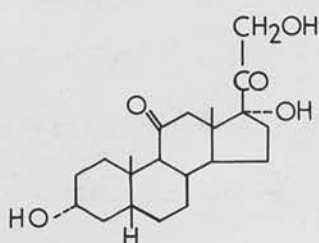
Figure 6: The conjugates of steroids with glucuronic and sulphuric acids.



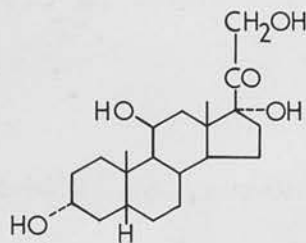
Cortisone



Hydrocortisone



Tetrahydrocortisone



Tetrahydrocortisol

Figure 7: Compounds with the dihydroxyacetone / α-Ketol side chain.

majority are conjugated with glucuronic acid and sulphuric acid, figure 6. Hydrolysis of these conjugates by boiling with strong mineral acids alone may not always be adequate from the quantitative point of view; furthermore it has been suggested that the steroids may form artifacts during the process of hydrolysis and extraction and may cause either an underestimate or an overestimate of the true 17-KS content in urine. Thus the usefulness of 17-KS estimation as an index of adrenal function is limited to the finding of a gross departure from the normal.

Method of Reduction.

During the last fifteen years or so, other chemical methods which aimed at a more direct assessment of adrenal function became available. Most of them depended on the different colour reactions produced by the functional groups present in the 'glucocorticoid' molecule. For analytical purposes, the all important factor is the type of side chain at C-17. Figure 7 shows the side chain carried by hydrocortisone, cortisone and their metabolites tetrahydrocortisol and tetrahydrocortisone. This is a dihydroxyacetone side chain of which the arrangement at C-20 - C-21 constitutes an α -ketol grouping.

The first attempt to estimate the urinary corticosteroids by making use of the reducing property of the α -ketol functional group was made by Talbot et al (1945). The reagent used to measure these 'reducing steroids' in urine was alkaline copper sulphate. Likewise, a year later, Heard and Sobel using phosphomolybdic acid reagent reported that this substitution enabled the reducing action of the α -ketol to be enhanced by the presence of the Δ^4 -3-ketone group. In 1951 Chen and Tewell, reporting their experiences with/

with a tetrazolium salt, 2:3:5- triphenyl tetrazolium chloride, proposed yet another analytical reagent for the measurement of these steroids. On reduction in alkaline medium, this compound produces a red formazane. More recently, a similar compound but twice as sensitive called blue tetrazolium has replaced the original.

The two original techniques measured only the free reducing steroids and did not take into account the conjugated metabolites which represented the majority fraction of steroids secreted by the adrenal cortex. And when later it was discovered that these techniques also measured other non steroidal reducing substances in the urine which could not be removed even with preliminary purification thereby causing serious errors in the estimation, these methods tended to fall into disuse. However, in recent years, a few workers have felt it worthwhile to attempt to improve these reducing methods and Raftopoulos et al (1959) have recorded a normal output of 7.8 - 13.2 mg/day for a molybdate method and 8.5 - 10.5 mg/day for a blue tetrazolium method - results which seem to be of the right order when compared with the most widely accepted of present day methods (see Table 1).

Oxidation Method.

Not long after the reduction methods had been proposed and come into use, another method based on a different line of approach became available. This new approach was initiated by Lowenstein et al (1946) and later improved by Daughaday et al (1948) and by Corcoran and Page (1948). These workers made use of the fact that corticosteroids with a hydroxyl group on C-21 and with or without the same group at C-17, react with periodic acid to yield 1 mole of formaldehyde per mole of corticosteroid. Figure 8 shows /

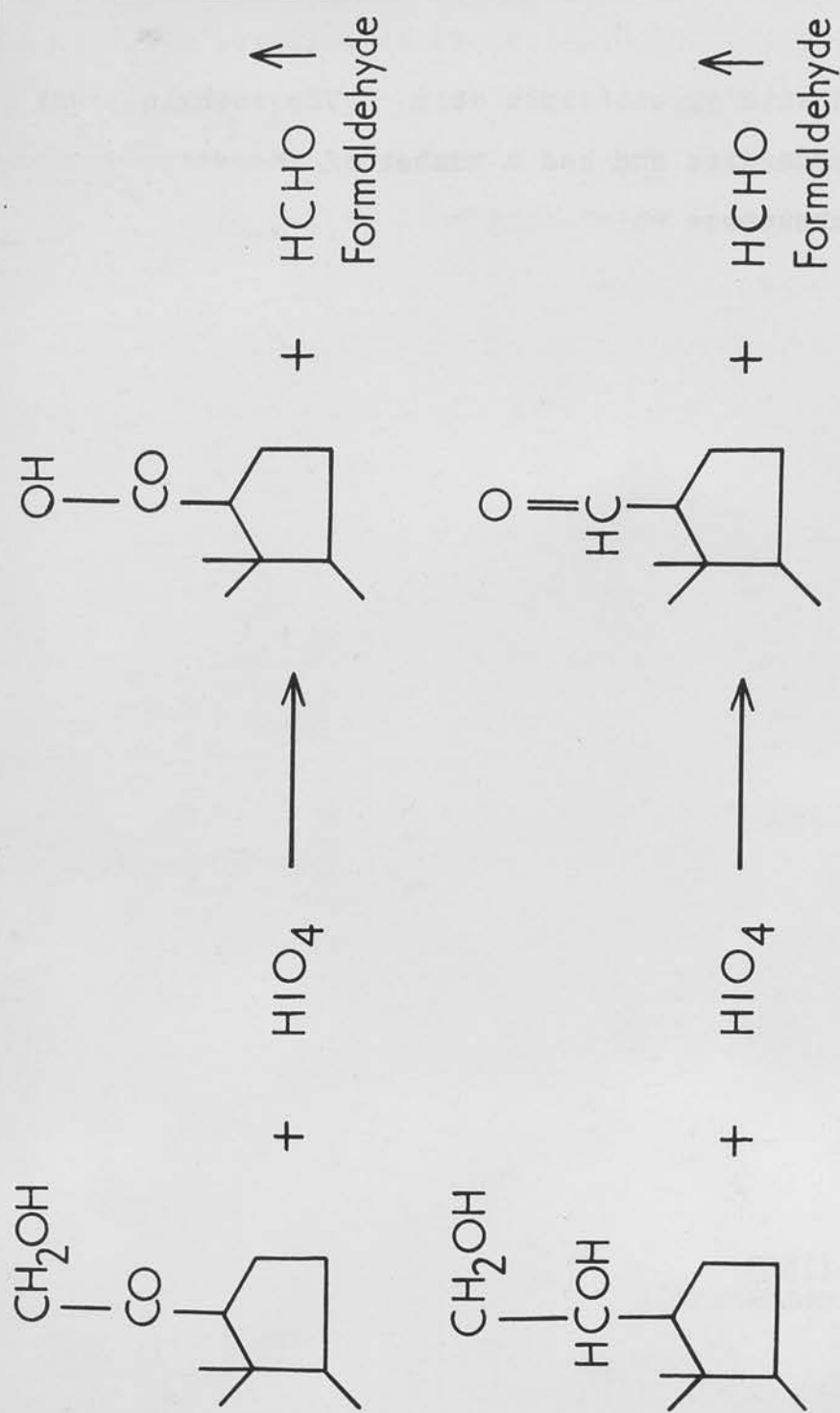


Figure 8: The reactions of formaldehydogenic steroids.

shows the mechanism of this reaction. After removal of the excess oxidant, the formaldehyde is liberated from the reaction mixture by steam distillation, trapped in sulphite solution and allowed to react with chromotropic acid. The technique was difficult to standardise and had a number of important drawbacks. Contaminating substances which could not be removed by preliminary washing also yielded formaldehyde on oxidation with periodic acid. Other urinary contaminants had a binding effect on formaldehyde inhibiting both its full liberation and its reaction with chromotropic acid. Formaldehydogenic steroids estimated after enzyme hydrolysis have yielded figures far in excess of those obtained by other methods. Data shown on Table 1 illustrates this point and because of this, the method is now little used.

Table 1

Urinary corticosteroid excretion values mg/24 hr.

	<u>Normal adult Males</u>	<u>Females</u>
Glenn and Nelson (1953) (enzyme, Porter Silber)	3.3-9.3	2.1-5.0
Reddy (1954) (Porter Silber, without hydrolysis)	2.9-12.0	1.1-8.6
Dunstan et al (1953) (enzyme, formaldehyde)	7.1-62	4.9-33
Sulkowitch et al (1955) (enzyme tetrazolium)	4.1-7.5	4.3-7.0

Porter Silber Colour Reaction.

Another method, more important than those so far discussed, and which employed the Porter-Silber colour reaction (1950) became available for routine use. In this method, the urinary corticosteroids are first hydrolysed with β -glucuronidase, extracted with chloroform or ethyl acetate and the extract purified by carefully controlled washing processes with or without subsequent chromatographic separation. The 'purified' corticosteroids with the dihydroxyacetone side chain in alcoholic solution are made to react with phenylhydrazine in the presence of sulphuric acid to produce a characteristic yellow colour with a maximum absorption at 410 m μ . Many workers, particularly in America have closely followed the Glenn and Nelson method (1953) in carrying out this type of estimation.

Reddy et al (1952) however, dispensed with the hydrolysis stage and extracted the free as well as the conjugated steroids with butanol. The butanol extract was subjected to a short step purification and the corticosteroids with the dihydroxyacetone function group estimated by application of the Porter-Silber colour reaction.

With proper attention to detail, published methods of estimating Porter-Silber chromogens provide a basis of a sound and accurate method of group analysis equal to or better than any routine 17-KS method in accuracy and reproducibility (Patterson 1960).

17-Ketogenic Steroids. (17-KGS)

It is now well recognised that only a small proportion of the hydrocortisone secreted appears as 17-ketosteroids in the urine. The great majority are excreted as tetrahydrocortisone and tetrahydrocortisol. In 1952, Norymberski proposed a method which measured this large group of hydrocortisone metabolites to reflect adrenal /

adrenal function. The side chain of these metabolites were oxidised with sodium bismuthate to generate production of 17-ketosteroids. Estimations of the urinary 17-KS were carried out before and after bismuthate oxidation and the difference was appropriately reported as 17-ketogenic steroids. The main advantage of this method lies in the fact that the oxidation of the labile side chain to the stable 17-KS group is carried out in 50% acetic acid before the conjugates are hydrolysed with acid.

In the opinion of Diczfalussy et al (1955) this method developed by Norymberski has the following advantages: (i) The technique circumvents the difficulties associated with hydrolysis of C-21 17-hydroxycorticosteroids. (ii) Normal excretion values are higher than those reported in the case of previously published methods. This is important in view of the fact that the reliability criteria of the technique are probably more satisfactory than those of previous methods. (iii) The procedure measures predominantly steroidal material and the components identified following chromatography are the expected derivatives of known 17-ketogenic steroids. (iv) The method is simple and is well suited for routine use in a clinical laboratory.

Blood Studies.

It is now well established that the adrenal cortex elaborates a large number of substances into the blood and that hydrocortisone is the predominant steroid. Corticosterone, which is a major steroid in some animals, is present in man in about one-tenth of the concentration of hydrocortisone; and aldosterone, although of great biological importance, is present in such quantities that special isotope techniques are necessary for its determination. And so/

so attention was paid to the measurement of plasma hydrocortisone and like substances to correlate plasma levels with adrenocortical activity. Some of the early methods and their results are summarized in Table 2.

The main problem particularly associated with plasma steroid determination is that of sensitivity since even the predominant steroid, hydrocortisone is only present in a concentration of about 10 ug%; and in addition to sensitivity there is also the problem of specificity. Consequently many of the methods which have been devised depend upon a preliminary separation of the steroids, often by chromatography, followed by a suitably sensitive reaction for the estimation.

Table 2

Early attempts to measure Plasma adrenal steroids

Authors	Method involving	Plasma steroids ug% Hydrocortisone or 17OHCS.
Nelson and Samuels (1952)	Chromatography and Porter-Silber reaction	4-10
Bondy and Altroek (1953)	17-OHCS; Porter-Silber	7.8 \pm 3.3
" " (1957)	Chromatography and Fluorescence	8.1 \pm 3.6
Morris and Williams (1953)	Chromatography and Polarography	8.4
Sweat (1954)	Chromatography and Fluorescence	10.8 \pm 2.6
Silber and Porter (1954)	Rapid method - Porter- Silber reaction	13.3 \pm 6.2
Peterson et al (1957)	Modified Silber Porter	14.0 \pm 4.5

One of the earliest methods applicable to the routine determination of plasma 17-hydroxycorticosteroids (17-OHCS) was that proposed by Nelson and Samuels (1952). Their method measured mainly hydrocortisone and depended on the presence of the dihydroxy-acetone functional group. They extracted plasma, chromatographed the extract on magnesium silicate and collected the third of the three eluate fractions. The third fraction containing the 17-OHCS - subsequently demonstrated to consist mainly of hydrocortisone by paper chromatography - was measured by the Porter-Silber colour reaction. With this method, normal blood levels ranged from 4 to 10 ug per 100 ml plasma. Recoveries of 1 to 4 ug of hydrocortisone added to 10 ml of plasma and run through the floresil column varied from 55% to 116%. This method has been used in many laboratories, particularly in the United States and when it became evident that reproducibility in the method was not always attained, various modifications of the original technique were proposed. In 1953, Eik-Nes, Nelson and Samuels published additional technical details and emphasised the importance of careful preparation of materials, particularly the chromatographic adsorbent, Florisil.

In 1953, Bondy and Altrock published a somewhat similar method for the analysis of 17-OHCS. It involved a complex extraction of plasma through several steps; the final extraction being made in a chloroform-carbon tetrachloride mixture which was then evaporated to dryness and the Porter-Silber colour reaction applied. From 60 to 70% of the hydrocortisone acetate was recovered when this steroid was added to the plasma. Their normal mean value was 7.8 ± 3.3 ug%. In a more recent method, Bondy et al (1957) used paper chromatography with fluorimetry to estimate plasma hydrocortisone. A correction /

correction for incomplete recovery was made using ^{14}C -labelled hydrocortisone and a normal level of $8.1 \pm 3.6 \text{ ug\%}$ was reported.

In the same year Morris and Williams (1953) published a method on the polarographic estimation of steroid hormones. These authors extracted heparinized blood with various solvents and the extracted steroids were fractionated by partition chromatography. The various fractions were then subjected to polarographic identification and quantitation. Among other steroids, recovery of added F to blood was 80% and in normal plasma the mean concentration of hydrocortisone was found to be 8.4 ug\% . This method has not received widespread favour because of its technical complexity.

In 1954, Sweat reported that hydrocortisone and corticosterone produced intense fluorescence in ethanolic-sulphuric acid solution and that the fluorescence was measurable in a Beckman spectrophotometer. From these studies the author made the following generalizations: (i) the presence of a hydroxyl group at C-11 seemed to be necessary for increased fluorescence and (ii) the presence of an alpha-beta unsaturated ketone in ring A also seemed to exert an influence on fluorescence. In 1955, Sweat published a method for the measurement of adrenocortical steroids in peripheral blood. Blood was extracted with chloroform and the extract was partitioned and chromatographed on silica gel. The eluate fraction containing hydrocortisone was then measured by sulphuric acid induced fluorescence. The average concentration of hydrocortisone in peripheral blood was reported as being 10.8 ug \% . When A.C.T.H. was given to normal subjects, there was a two to five fold increase in hydrocortisone like steroids in the blood. When A.C.T.H. was given to Addisonian patients, there was no significant increase in the level of blood steroids as determined by fluorescence.

In 1954, Silber and Porter proposed another method for the determination of hydrocortisone in plasma. Their method dispensed with the need for chromatography and involved extraction of plasma to which hydrocortisone (5ug) was added to allow for any loss of steroid in the extraction procedure; this amount was subtracted at the end of the analysis. An attempt was also made to diminish the number of steps in the procedure so as to minimise the loss of steroids. Thus the alcoholic - phenylhydrazine - sulphuric acid reagent was added directly to the chloroform extract. The steroid passed directly from the chloroform into the reagent and after removal of the chloroform the amount of steroid present was estimated colorimetrically. The average plasma level in normal subjects was 13.3 ug%. Colour reaction took place at room temperature, was complete in about 8 hours and remained stable for another 12 hours. The method appears to be good and readily adaptable to routine use. Peterson et al (1957) have modified this procedure, further simplifying it and increasing its sensitivity.

Several methods are now available for the estimation of plasma hydrocortisone. As a rule they describe advantageous modifications of those methods cited above. Only very recently, working in this laboratory, Osman (1961) developed a rapid method for the simultaneous determination of hydrocortisone and corticosterone starting with as little as 1-3 ml of plasma. Dispensing with paper chromatography but making use of two different sulphuric acid concentrations, this worker measured and plotted the intensities of the acid induced fluorescence of the plasma extract over a short period of time. Then by making reference to the fluorescence curves of authentic hydrocortisone and corticosterone and employment of the proposed algebraic expression, the plasma levels of hydro-/

hydrocortisone and corticosterone were determined.

Hydrocortisone Production Rate.

In general, hyperactivity or hypoactivity of the adrenal cortex is reflected by increased or decreased plasma concentrations of hydrocortisone and excretions of its metabolites. However there may be conditions in which the output of the adrenal cortex is altered without demonstrable changes in the corticosteroid content of blood or urine. Thus the actual amount of hydrocortisone elaborated by the adrenal cortex has been the subject of a great deal of speculation and resulted in some attempts being made, see Table 3.

Knowlton in 1952, in treating 14 cases of Addison's disease found that a replacement therapy of 12.5-25 mg of cortisone per day was quite adequate, suggesting that the daily output of the normal gland was of this order.

Several attempts have been made to correlate the excretion of certain groups of hydrocortisone metabolites with the actual production rate by observing the proportion of an ingested dose which is subsequently excreted in the form of such groups of compounds. Thus Dorfman (1954), from investigating eight normal male adults between the ages of 19-35 and six normal female adults between the ages of 21-35, calculated the normal production rate of hydrocortisone to be 18-21 mg/day on the basis of the quantities 11-oxy and 11-deoxy-C-19 neutral 17-ketosteroids in the urine.

Silber (1955) administered graded doses of hydrocortisone to 4 subjects and by comparing the amount of metabolite excreted after the test dose with that excreted during the control or basal period, used the fraction obtained to calculate the hydrocortisone production rate which indicated an average of 13.8mg/8 hrs (8.30am-4.30pm) as/

as judged from the quantity of endogenously produced phenylhydrazine reactive chromogens in the dichloromethane extracts of glucuronidase hydrolysable urine. When the same procedure was applied to 20 male (21-35 years of age) and 24-hour urine collected instead of 8-hr urine the adrenal output was calculated to be 21.9 ± 8.2 mg/day.

Table 3

Authors	Method based on	F.Prod.Rate in Normals mg/day
Knowlton (1952)	Replacement therapy	12.5-25
Dorfman (1954)	Excretion of 11-oxy and 11-deoxy neutral 17-KS	18-21
Silber (1955)	17-OHCS, Excretion after test dose	21.9 ± 8.3
Bondy and Altrock (1953)	Simultaneous collection of left renal vein and renal artery blood 17-OHCS	15-25
Moxham and Nabarro (1956)	Correlated oral dose with excretion of a) 17-KGS b) 17-OHCS	a) 5.5-49 b) 9.5-45
Samuels et al (1957)	Rate of disappearance of adm. hydrocortisone	36
Peterson and Wyngaarden (1956)	Isotope Dilution (blood method)	17 - 29
Cope and Black (1958)	Isotope Dilution (urine)	5 - 25

In 1956 Moxham and Nabarro, using their own modification of the methods of Norymberski for 17-KGS and that of Reddy et al for 17-OHCS, correlated oral hydrocortisone dosage with both ketogenic and 17-OHCS excretion. They thereby provided data which suggested a production of between 5.5 and 49mg/day as judged by 17-KGS and 9.5-45mg/day as judged by the 17-OHCS.

In 1953, Bondy and Altrock, by simultaneously collecting samples of left renal vein and renal artery blood, determined the difference of hormone secretion by a method of analysis of 17-OHCS. When the difference was multiplied by the value of renal plasma flow, the calculation revealed a production rate of 15 to 25 mg per day. Although a method like this indicates a more direct estimate, experimentally the procedure is difficult to perform and therefore has very limited application.

Samuels et al (1957) employing both the plasma hydrocortisone level and the rate of disappearance of infused hydrocortisone obtained an average production rate of 21.8 ug/KG/hr. This works out to about 36 mg/day for normals between the ages of 33-55 yrs.

The analytical procedures thus far described in the study of hydrocortisone production rate may be hampered by incomplete enzymatic hydrolysis and incomplete solvent extraction. In an attempt to obtain a solution to these problems and to provide a more reliable and accurate estimate of the average rate of hydrocortisone production in man, some workers have used isotopically labelled hydrocortisone. In this procedure, a trace quantity of the radioactive material is administered either orally or intravenously and the subsequent measurement of its rate of dilution with the hydrocortisone produced by the subjects' own adrenals is determined by comparing the specific activity of the administered dose with that at the time of complete mixing. Production rate of hydrocortisone is then calculated by conventional isotope dilution expressions with analytical errors considerably reduced since a procedure of this nature does not depend upon complete recovery.

Peterson and Wyngaarden in 1956 and also Cope and Black (1958)/

used this isotope dilution technique to assess adrenocortical function - the former from blood studies and the latter from urine studies. In this research both these methods have been used and the detailed procedures are described in the next chapter.

Peterson and Wyngaarden employed the gas flow type of counter, while Cope and Black employed the end window Geiger-Muller counter for radioactivity measurements. In this research, activity was measured by employment of a Panax Liquid Scintillation counter and thus the first task undertaken was the establishment of the two methods using this new type of counter.

Using both the blood and urine methods, normal production rate patterns were determined. Investigations were then carried out on patients with various endocrine disorders - particularly those with Cushing's disease and those with Hirsutism. The results and discussion of these investigations are presented in Chapter III. In most cases besides hydrocortisone production rate studies, other tests of adrenal function - 17-KS, 17-KGS - were at the same time carried out; so that in the light of the clinical picture presented, a good comparison may be made of the various laboratory tests normally carried out for adrenal function.

In the study of the role of the adrenal gland in the metabolic response to surgery, the actual increase in production rate in response to surgical stress seemed worthwhile investigating together with balance studies of sodium, potassium and nitrogen before and after operation. Chapter IV briefly introduces this fascinating line of research and some data concerning the hydrocortisone production and electrolyte alterations following surgery are presented.

Chapter II : Description of Methods Used

Method of Hydrocortisone Production Rate from Blood Studies

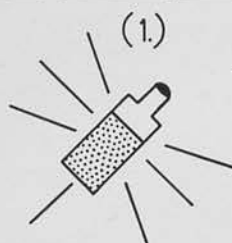
The method to be described is based largely on that described by Peterson and Wyngaarden (1956). A dose equivalent to 0.2 μ c, instead of Peterson's 1 to 2 μ c, constitutes the amount of radioactivity administered and 5 x 25 mls of blood are withdrawn in the course of the experiment instead of 6 x 60 ml.

Principle.

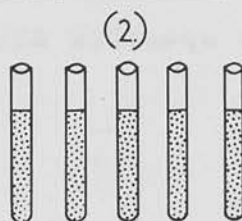
When a small dose of ^{14}C labelled hydrocortisone is injected intravenously, it promptly 'mixes' with the hydrocortisone present in the body. If by some means it is possible to measure the degree of dilution of the administered dose with the hydrocortisone produced by the body, the reservoir or miscible pool of hydrocortisone in the body may be calculated. This can actually be done by making serial determinations of the rapidly falling specific activity of plasma hydrocortisone at definite intervals after the administration of the dose. The plot of the specific activities against time of withdrawal of blood on a semilog graph reveals a straight line which extrapolated to zero time provides the specific activity of plasma hydrocortisone at the theoretical time of complete mixing. By comparing this specific activity with that of the administered dose, the degree of dilution may be calculated. Also from the graph, by consideration of the half clearance time, the turnover may be obtained and by making use of the conventional isotope dilution expressions, the miscible pool and turnover rate of hydrocortisone can be calculated.

Figure 9 attempts to show at one glance the important steps involved in the method :

Figure 9: Main steps in the measurement of production rate from blood.



Dose adm. at 9.30am.

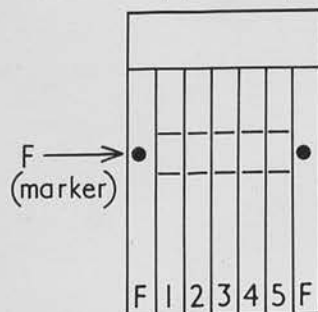


25ml. blood withdrawn
every 1/2 hr. for 2 1/2 hrs.

(3.)

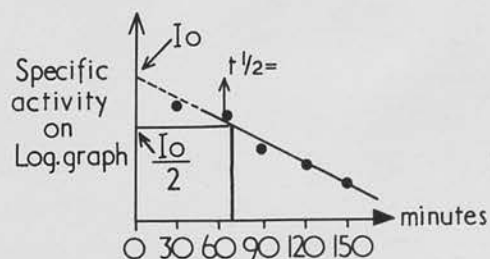
CHROMATOGRAPHY

Isolation of plasma hydrocortisone



Cut, elute and measure the
5 specific activities (SAs)

(4.)



Plot to give SAs vs time.
Extrapolate to give I_0 and $t_{1/2}$

(5.) CALCULATION

$$A = a \left[\frac{I_1}{I_0} - 1 \right]$$

$$K = \frac{0.693}{t_{1/2}}$$

$$\underline{F \text{ production rate}} = \frac{Ax}{\Lambda} = K \times 24$$

1. The dose is administered intravenously at 9.30 AM
2. Heparinized blood is withdrawn every $\frac{1}{2}$ hour for $2\frac{1}{2}$ hours.
3. Plasma hydrocortisone is isolated by paper chromatography
4. The specific activities of the five eluted spots are determined and plotted against time on a semilog graph. Extrapolation provides the specific activity at zero time and by application of the isotope dilution expression, the production rate of hydrocortisone is calculated.

Reagents required :

1. Methylene chloride : BDH analar grade
2. Benzene : " " "
3. Methanol : " " "
4. Sodium Hydroxide : N/10 approximately
5. Acetic Acid : "
6. Sulphuric Acid : 80% (with dist. H₂O)
7. Hydrocortisone Markers : 2mg/ml in alcohol
10 μ L \equiv 20 μ g to be used for chromatography
8. Hydrocortisone Standard : 1 μ g/ml in alcohol
.2ml \equiv .2 μ g to be used for fluorimetry
9. Purified Alcohol : 500 ml are treated with 40 pellets of KOH and refluxed for 2 hours. On gentle distillation the middle fraction is collected.

Method.

Administration of the dose.

5.0ml of the dose solution (equivalent to 0.2 μ c and of known specific activity and quantity of hydrocortisone) were carefully pipetted into a sterile beaker and the contents withdrawn into a syringe. The beaker was rinsed with physiological saline and /

and the washings also withdrawn into the syringe. The dose was administered intravenously over a period of three minutes. When the injection was complete, blood was withdrawn into the barrel of the syringe and again pushed back into the blood stream to ensure completeness of administration of the dose.

Collection of Blood Specimens.

25ml of blood were withdrawn in a heparinized syringe every half hour for two and a half hours after administration - the exact time of withdrawal being noted. Plasma was separated from the red cells as soon as possible.

Extraction.

From each specimen, 10ml of plasma were extracted with 100ml of methylene chloride by gentle continuous rotation for 10 minutes, then centrifuged for 10 minutes at 2000 r.p.m. The upper aqueous layer was removed by suction and the extract was washed once with 15ml N/10 NaOH, once with 15ml N/10 CH₃COOH and once with 15ml distilled H₂O; removal of the aqueous layer each time being by suction after centrifugation. The washed extract was evaporated to dryness under reduced pressure and in a water bath at 40°C. 10ml of distilled water treated in the same manner as plasma served as a blank extract.

Isolation of Hydrocortisone.

Fractionation of the plasma steroids and isolation of hydrocortisone was brought about by paper chromatography using the Bush₅, benzene/methanol water system. Each extract was redissolved in 2 x 50 μ L methylene chloride and transferred to the starting line of the washed No 1 Whatman chromatography paper. The papers/

papers were placed in the chromatography tank and allowed to equilibrate overnight. On the next morning, benzene - saturated with the stationary phase - was introduced and descending chromatography was allowed to proceed. At the end of a 5 to 6 hour run, the papers were withdrawn from the tank and left to dry in the dark for $\frac{1}{2}$ hr. The spots containing the 20 μ g hydrocortisone markers, which were applied at the same time as the extracts, were located under the ultraviolet lamp. The zones containing the extract hydrocortisone and blank on the other limbs of the paper were cut out at a position directly opposite those of the markers, fluted and eluted overnight by immersion using 5 ml of ethanol.

Determination of Specific Activity.

From each eluate, 3ml were taken for radioactivity counting and 0.5ml (or 1ml) for sulphuric acid induced fluorimetric estimation of hydrocortisone. The total activity expressed as counts per minute (cpm) found in the 5ml eluate divided by the amount of hydrocortisone expressed as μ g found in the same 5ml eluate provided the specific activity of the sample.

$$\text{i.e. Specific Activity} = \frac{\text{cpm in 5ml eluate}}{\mu\text{gF in 5ml eluate}} = \text{cpm}/\mu\text{g.}$$

The specific activities of the 5 specimens thus determined were plotted against time of withdrawal of the specimen on a semilogarithmic graph. The line of fit containing the points on the graph was extrapolated to zero time to give the specific activity at the theoretical time of complete mixing and the biological half life.

Calculation.

The miscible pool or reservoir of body hydrocortisone may be calculated from the isotope dilution expression : /

expression :

$$A = a \left[\frac{I_i}{I_o} - 1 \right]$$

where A = the miscible pool

a = the amount of F in μg in the administered dose

I_i = the specific activity of the dose in cpm/ μg

I_o = the specific activity at zero time.

The rate at which the miscible pool A is replaced with newly synthesized hydrocortisone from the adrenal cortex is obtained from

$$K = \frac{0.693}{t_{\frac{1}{2}}} \text{ pool/hr}$$

where K = the turnover constant and $t_{\frac{1}{2}}$ = the biological half life expressed in hours.

If A is expressed in mg., then the product of A and K provides the turnover of hydrocortisone in mg/hr which multiplied by 24 gives the production rate in mg/day.

Example.

If the amount of hydrocortisone administered (a) was 10 μg , the specific activity of the adm. dose (I_i) was 21000cpm/ μg and the specific activity at zero time (I_o) was found to be 210cpm/ μg then the miscible pool :

$$\begin{aligned} A &= 10 \left[\frac{21000}{210} - 1 \right] \mu\text{g} \\ &= 0.99 \text{ mg} \end{aligned}$$

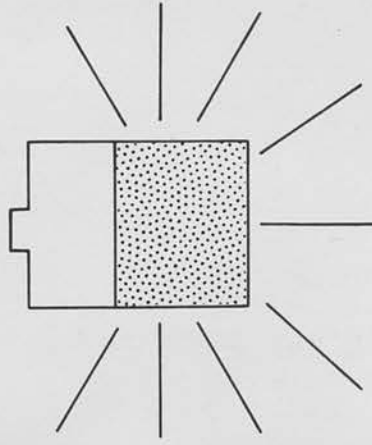
And if the biological half life $t_{\frac{1}{2}}$ was found to be 60 minutes (or 1hr), then

$$\begin{aligned} K &= \frac{0.693}{1} = .693 \text{ pool/hr} \\ AK &= 0.99 \times 0.693 \text{ mg/hr} \end{aligned}$$

$$\begin{aligned} \text{Production Rate} &= AK \times 24 = 0.99 \times 0.693 \times 24 \\ &= 16.3 \text{ mg/day.} \end{aligned}$$

Figure 10: Main steps in the measurement of production rate from urine.

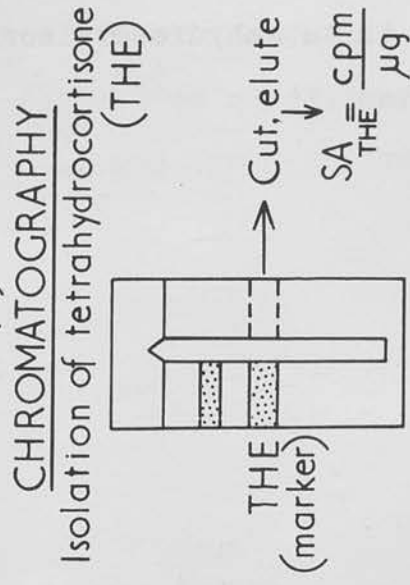
(1.)



Collect 24 hour urine after administration of dose.

Total Radioactivity Count = Cu.

(2)



CALCULATION

$$(3) \text{ F_production rate} = \frac{Cu}{SA_F} \times \frac{SA_F}{SA_{THE}} = \frac{Cu}{SA_{THE}}$$

Measurement of Production Rate from Urine Studies.

The measurement of hydrocortisone production rate from urine is, in principle, the same as that from blood. In urine, the main metabolite of hydrocortisone is tetrahydrocortisone, so that to calculate the degree of dilution, it is convenient to determine the specific activity of this metabolite and compare it with that of the administered dose. It will be shown later that all that is necessary to calculate the production rate from urine is a) the measurement of the total radioactivity in the 24 hour urine and b) the measurement of the specific activity of the urinary tetrahydrocortisone.

Figure 10, like figure 9, attempts to show at one glance the principle steps involved in this method:

1. Measurement of the total activity in the 24 hour urine.
2. Isolation of the urinary tetrahydrocortisone by paper chromatography after enzymatic hydrolysis with β -glucuronidase and determination of its specific activity.
3. Calculation of production rate by isotope dilution using data obtained from steps 1 and 2.

This method is based largely on that described by Cope and Black (1958); the only modification being the use of Nowaczynski's ethylene glycol/toluene system instead of the original Zaffaroni's propylene glycol/toluene system in the paper chromatographic separation of the urinary steroids.

Reagents required :

1. β -glucuronidase : (limpet)
2. Acetate Buffer : 0.5M, pH 4.6
3. Chloroform : M & B Lab. Chemicals

4. Sodium Hydroxide : N/10 approximately
5. Toluene : B.D.H. Analar Grade
6. Ethylene Glycol : B.D.H.
7. Methanol : B.D.H. analar grade
8. Alcohol : Purified
9. Blue Tetrazolium : B.D.H.
10. Tetra methylammonium hydroxide : B.D.H.
11. Hydrocortisone standard : 100/ μ g/ml in ethanol
i.e. 0.2ml \equiv 20 μ g.

Method

Administration and Urine Collection.

The subject under investigation was asked to empty his bladder first and the dose was administered either orally or intravenously. 24 hour urine was collected without preservative. An aliquot of urine was taken for radioactivity counting and after correcting for quenching by employment of an internal standard, the total activity in the 24 hour urine was calculated and expressed as counts per minute (cpm).

Enzymatic Hydrolysis.

Two lots of $\frac{1}{10}$ total volume of urine were brought to pH 4.6 by the addition of 1N HCl and stabilised with 100ml of 0.5M acetate buffer, pH 4.6. A quantity, equivalent to 500 units per ml of urine, of β -glucuronidase was dissolved in some acetate buffer solution and added to the buffered urine. Both specimens were then incubated for 24 hours at 37°C.

Extraction and Purification.

Extraction of the urinary steroids was carried out using 2x50ml chloroform for each sample and the emulsion formed during the extraction was broken by centrifugation for 15 minutes at 2000 r.p.m. The chloroform extracts were washed twice with 15ml N/10 NaOH to remove phenolic, acidic pigmented substances and once with 15ml distilled water. The washed extracts were brought to dryness under reduced pressure and on a water bath at 50°C.

Chromatography.

The fractionation of the urinary steroids and the isolation of the tetrahydrocortisone were brought about by paper chromatography and the employment of the ethylene glycol/toluene solvent system. Washed Whatman paper No.54 was used. The paper was dipped into the stationary phase solution containing ethylene glycol: methanol (1:1) and the excess solution removed by pressing the wet paper between two sheets of dry Whatman paper. The extracts containing the steroids were taken up with 2x.1 ml chloroform and transferred to the starting line on the paper carrying two limbs (one for each extract). The paper was then transferred to the chromatographic tank and as equilibration was not necessary, the mobile phase - toluene saturated with ethylene glycol - was introduced. During the run which took 14-16 hours, the tank was kept in the dark in a cupboard. After the run the paper was taken out of the tank and left to dry. This is difficult and normally takes a long time owing to the presence of ethylene glycol, but drying may be hastened by employment of a hair dryer or by hanging the strips in a drying cabinet operating at about 50°C.

Location and Elution.

To locate the tetrahydrocortisone zone, one of the limbs on the paper was cut out and developed by dipping it into a solution containing 1 part of 0.5% blue tetrazolium in water and 3 parts of 10% NaOH in 60% aqueous methanol. In a few seconds two distinct purplish mauve bands appeared. The one nearer the origin was due to the water insoluble formazan formed by the interaction of the tetrahydrocortisol with the blue tetrazolium and the other which was more intense and further away from the origin was that due to the tetrahydrocortisone. On the other limb, a section opposite to the developed tetrahydrocortisone band was cut out, fluted and eluted by immersion in 5ml ethanol overnight.

Specific Activity of the THE

3.0ml of the eluate were taken for radioactivity counting and the total activity contained in the 5.0ml eluate was calculated. To determine the concentration of tetrahydrocortisone in the 5.0ml eluate, 0.5ml eluate was taken and 3.2ml ethanol were added to it. To this, 0.1ml of .5% BTZ in alcohol and 0.2ml of .1% tetramethylammonium hydroxide were added. At the same time, 0.2ml ($\approx 20\mu\text{gF}$) hydrocortisone standard was added to 3.5ml alcohol and treated likewise as the eluate (i.e. + .1ml BTZ solution + .2ml tetramethylammonium hydroxide solution). Lastly, 3.7ml alcohol, also treated in the same way as the unknown and standard, served as a blank. The solutions were well mixed and kept in the dark for 90 minutes. The optical densities were read at a wavelength of 520 m μ on an SP600 against an alcohol blank. The amount of THE was calculated, multiplied by 10 and expressed as $\mu\text{g}/5\text{ml}$ eluate. The /

The specific activity of the THE was obtained by dividing the radioactivity contained in 5ml eluate by the amount of THE in the same 5ml eluate.

$$\text{i.e. Specific Activity} = \frac{\text{cpm in 5ml eluate}}{\mu\text{g in the 5ml eluate}} = \frac{\text{cpm}}{\mu\text{g}}$$

Calculation.

If Cu be the total count in the 24hr urine, and SA_F the specific activity of the administered dose, the ratio of Cu/SA_F represents the amount of radioactive hydrocortisone present in the urine in the form of all metabolites. And if SA_U be the specific activity of the urine metabolites, the ratio SA_F/SA_U represents the degree of dilution of the administered hydrocortisone with the hydrocortisone produced endogenously, provided the administered radioactive hydrocortisone is metabolised in exactly the same way as the hydrocortisone produced in the body. In practice it will not be necessary to measure the specific activity of all the hydrocortisone metabolites; the determination of the specific activity of tetrahydrocortisone (SA_{THE}) is not only representative but also convenient. Thus

$$\text{Hydrocortisone Production Rate} = \frac{Cu}{SA_F} \times \frac{SA_F}{SA_{THE}} = \frac{Cu}{SA_{THE}}$$

Example.

If the total 24 hour urine volume is 1000ml, and the activity contained in 0.5ml urine is 90 cpm (corrected for quenching), then the total activity in the 24hr urine (Cu) = 180,000cpm.

Again if the activity in the 5ml eluate is 900 cpm and the amount of THE in the 5ml eluate is 50 μg , then the specific activity of THE (SA_{THE}) = $\frac{900}{50} = 18 \text{ cpm}/\mu\text{g}$.

$$\begin{aligned}\text{Hydrocortisone Production Rate} &= \frac{\text{Cu}}{\text{SA}_{\text{THE}}} = \frac{180000}{18 \times 1000} \text{ mg} \\ &= 10 \text{ mg/day}\end{aligned}$$

Urinary 17-Ketogenic Steroids.

This familiar and reliable laboratory test for adrenal function was carried out by a rapid micro method based on that of Dreker et al (1952) in which ethylene dichloride was used as the extracting solvent. The urinary 17-ketosteroids were determined before and after bismuthate oxidation and the difference provided the 17-ketogenic steroids.

(a) 17-Ketosteroids.

16ml of urine were pipetted into a quickfit centrifuge tube of about 50ml capacity and to this, 6ml concentrated HCl were added and the whole placed in a boiling water bath for 10 minutes. At the end of the hydrolysis, the contents were cooled in water and 20ml of ethylene dichloride were added. The tube was well stoppered and shaken mechanically for 15 minutes. The contents were then centrifuged at 2000 r.p.m. for 10 minutes and the aqueous phase removed by suction. To remove the acidic and phenolic substances and pigments, the ethylene dichloride extract was shaken with 5ml distilled water for 2 minutes, then with 5ml of 3N sodium hydroxide for 5 minutes. After each washing, the layers were separated by centrifugation and the upper aqueous phase removed by suction. The washed extract was filtered through fluted No.1 filter paper into a test tube. 10ml of the filtrate were transferred into a stoppered test tube and brought to dryness under reduced pressure. The dry residue was dissolved in 0.2ml alcohol/

alcohol and for colour development, the following tubes were set up :

1. The unknown :

- 0.2ml alcohol containing the urine extract
- 0.2ml of 2% m-dinitrobenzene in alcohol (freshly prepared)
- 0.2ml of 2.5N alcoholic KOH (freshly prepared)

2. The standard :

- 0.2ml alcoholic solution containing 0.1mg dehydroisoandrosterone
- 0.2ml of 2% m-dinitrobenzene in alcohol
- 0.2ml of 2.5N alcoholic KOH

3. The reagent blank :

- 0.2ml alcohol
- 0.2ml of 2% m-dinitrobenzene in alcohol
- 0.2ml of 2.5N alcoholic KOH

The tubes were stoppered and placed in a bath at 25°C in the dark for 1 hour. Then to each tube, 10ml of alcohol were added and the contents well mixed. The optical densities were read against an alcohol blank in an SP600 at wavelengths of 520 mμ and 430 mμ. Readings were made at 2 wavelengths so that a correction might be made for interfering chromogens.

$$\text{Corrected reading of unknown: } \frac{\text{Reading at 520m}\mu - 0.6 \times \text{Reading at 430m}\mu}{0.73}$$

$$\text{Mg. 17-ketosteroids/24hr urine} = \frac{\text{corrected reading at 520m}\mu \times 0.1 \times \text{Vol. 24hr}}{\text{Reading of standard} \times 8}$$

(b) Total 17-Ketosteroids.

To 4ml of urine in a stoppered test tube, 4ml of glacial acetic acid and 1.0gm of sodium bismuthate were added and the whole shaken mechanically for half an hour away from direct sunlight. Then 10ml of 12% sodium metabisulphite were added and shaken to reduce /

reduce the excess bismuthate. 6ml of concentrated HCl were added and the whole placed in a boiling water bath for 10 minutes. The contents were then cooled and extracted with 20ml of ethylene dichloride. The extract was washed with water and alkali and filtered into a test tube. 10ml of the filtrate were transferred to another test tube and evaporated to dryness under reduced pressure. As before

$$\text{mg. Total 17-KS/24hr} = \frac{\text{Corrected reading at 520m}\mu}{\text{Reading of std.}} \times 0.1 \times \frac{\text{Vol. 24hr}}{2}$$

$$\text{mg. 17-ketogenic steroids} = \text{Total 17-KS (b)} - \text{17-KS (a)}$$

Radioactivity, Chromatography and Fluorimetry etc.
as applied to the methods used.

Radioactivity.

^{14}C is a weak β -emitter and its measurement is therefore limited by the low energy of the β -particles ($\beta = 0.155 \text{ MeV}$). If an end window or windowless gas flow counter is used which involves plating of the source at either infinite thinness or infinite thickness and operating in the proportional or Geiger region, the sensitivity is low and there are the wellknown difficulties in obtaining reproducible plating of the material to be assayed. Correction has to be made for self-absorption by reference to a pre-calibrated self absorption curve.

The assay of ^{14}C in the gas phase after combustion of the biological material overcomes the difficulty of plating the samples and low sensitivity associated with end window counters. Counting efficiency is almost 100% due to the elimination of self absorption losses but the technique is laborious and requires considerable skill in operation. As an ordinary clinical laboratory tool, however, the disadvantages are too great.

The liquid scintillation counting of ^{14}C , in which the radioactive material is incorporated into a liquid phosphor and the scintillations "observed" by means of a photomultiplier, is gaining increasing popularity because of its high sensitivity, good reproducibility and the elimination of the window absorption, the necessity for plating and the elaborate technique required for gas counting.

Developments of liquid-scintillation counting as a practical method for the assay of radioisotopes in biological samples followed the work of Reynolds, Harrison & Salvini (1950) and Kallman & Furst (1950). Reviews of progress and development in liquid scintillation counting have been given by Davidson and Fiegelson (1957) and Bell and Hayes (1958). Guinn (1958) and Stitch (1959) have reviewed the advantages and disadvantages of liquid scintillation counting. The advantages cited include all those which have already been mentioned and amongst the disadvantages are the high cost of the complex apparatus, the lowering of the efficiency caused by quenching and the limitation of its use to samples soluble in the liquid phosphor. In this research all counting of ^{14}C was carried out with a Liquid Scintillation Counter.

Apparatus.

The assembly (Type SC/LP, Panax Equipment Ltd.) for scintillation counting of ^{14}C consisted of a lead castle, amplifier (Type 4250) with a gain of $\times 1000$ and an automatic counter (Type AC 300/6). Tap water was passed continually through the cooling coils of the head unit. The activity of solutions were measured in pyrex containers fitted with caps and with a capacity to hold a maximum of 15ml of solution. Optical contact between the window /

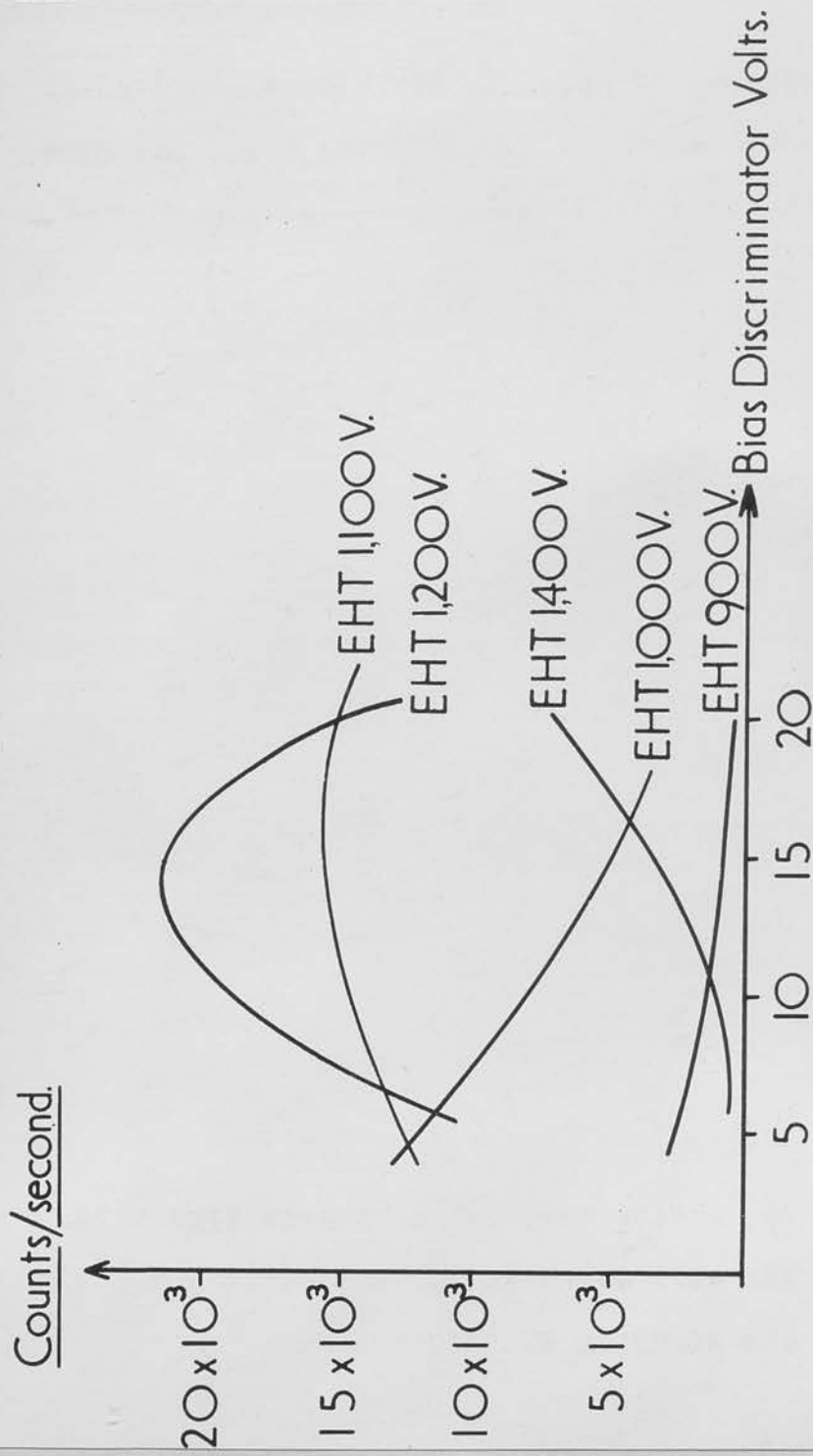


Figure 11: Five S^2/B curves of varying EHT's on Count rate VS Bias.
 Selected working voltages for C , EHT 1,200V, Bias 12V.

window of the counting vessel and the photomultiplier tube was maintained by the use of silicone oil (MS200/20).

Radiochemicals.

4- ^{14}C hydrocortisone of specific activity 21.7 $\mu\text{c}/\text{mg}$ was obtained free from the National Institutes of Health; Bethesda, Maryland, U.S.A. This material arrived in sealed ampoules each containing 5 μc and in a solution of 95% benzene and 5% methanol.

Organic Scintillators.

The choice of a suitable phosphor is very much a matter of personal choice. The author preferred the use of TTP/4 organic scintillator based on Toluene simply because it was cheaper and kept well.

Optimum working Conditions.

As this equipment was being used for the first time, it became necessary to determine the optimum working conditions for the counting of ^{14}C isotopes. In particular the extra high tension (EHT) and discriminator bias voltages which gave the greatest efficiency had to be determined. This was carried out by recording the background and count rate of a ^{14}C -source at each of a number of EHT settings (900-1600v) against a few bias discriminator voltages (5-20v). Figure 11 shows 5 source $^2/\text{background}$ (S^2/B) curves from which working voltages of 1200v and 12v were selected for the EHT and discriminator bias respectively for the counting of ^{14}C .

Preparation of the Dose.

The ampoule was carefully cut with a file and the contents equivalent to 5 μc were transferred by means of a clean syringe into /

into a Quickfit (B24, 50ml) test tube. The ampoule was rinsed twice with 1ml methanol and the washings added to the test tube containing the radioactive material. The pooled solution was evaporated to dryness under reduced pressure using the water pump and bath at 40°C. The dried extract was redissolved in 5ml alcohol and this solution represented the stock solution. One ml. of this stock solution, equivalent to about 1 μ c was diluted with 19ml of sterile distilled water so that 4 to 5ml of this 5% ethanolic solution containing 0.2 μ c served as one dose. To evaluate the specific activity of this dose solution, 0.2ml were taken for radioactivity counting and the same amount for fluorimetric quantitation of hydrocortisone content. So that if 5ml of this solution were administered, its total activity, specific activity and amount of hydrocortisone present had all been pre determined.

Counting Procedure.

In all counting procedures, 5ml of the toluene phosphor were always blended with 4ml of alcohol. The active solutions were brought to the same temperature as that in the castle by standing the glass containers holding them on running tap water and in the dark. When the sample was housed in the castle, a further 8-10 minutes were allowed for dark adaption before counting commenced. The proportion for the various types of samples for counting were as follows :

	<u>Toluene Phosphor</u> <u>ml.</u>		<u>Alcohol</u> <u>ml.</u>		<u>Active Sample</u> <u>ml.</u>
Background	5	+	4	+	-
Dose	5	"	3.8	"	0.2 (in alcohol)
Eluate	5	"	1	"	3 (in alcohol)

The background was counted until 4000 counts were recorded and the active samples until 10000 counts or a counting period of 1 hour - whichever was the faster. The dose solution and eluates containing the urinary tetrahydrocortisone were very active samples and 10000 counts were recorded in a few minutes; but the eluates containing the plasma hydrocortisone contained relatively much smaller amounts of radioactivity which decreased progressively from the 1st specimen to the 5th specimen and consequently required a much longer time to count.

With all liquid scintillation counting, quenching tends to lower the counting efficiency. Several investigators have studied the effect on counting efficiency of organic solvents commonly used in biochemical investigations, (Guinn 1958; Stitch 1959; Brown and Bradman 1961). Diethyl ether and ethanol have been reported to have only a slight quenching effect; but acetone and chloroform depress the efficiency considerably. Aqueous solutions, particularly urine - because of the pigments and dissolved salts - may sometimes exert a considerable quenching effect. To correct for quenching when counting urine an internal standard of known count rate was used. Counting of the urine was carried out before and after the addition of the standard; the observed count rate of the standard in urine was compared with its true value and so a correction factor to correct for quenching could be obtained.

To count urine, the solutions were prepared as follows :

	<u>Toluene Phosphor</u> <u>ml.</u>	<u>Alcohol</u> <u>ml.</u>	<u>Urine</u> <u>ml.</u>	<u>Int.Std.</u> <u>ml.</u>
Background	5	4	-	-
Urine sample	5	4	0.5	-
Urine + Std.	5	3.8	0.5	0.2 (in alcoholic solution = about 100cpm)

Example.

If the urine sample (minus background) registered 90cpm and the urine + standard registered 170cpm, then the observed count rate of the standard was (170-90)cpm. If the true count rate of the standard (.2ml) registered, say, 100 cpm, then the corrected count rate of the urine sample was

$$90 \times \frac{100}{170-90} = 115\text{cpm}$$

Urine does not mix with toluene but blending the phosphor with 4ml of alcohol, up to 0.5ml urine may be incorporated. Good mixing is ensured by gentle swirling for about 10 seconds.

Counting Efficiency.

With the exception of the eluates containing the plasma hydrocortisone, all counting carried out gave an accuracy with a standard error of 3% or less. The counting of the radioactively weak plasma samples gave an efficiency with standard errors ranging from 4% for the first sample to 10% for the last sample. The count rate of the background had always been about 60 c.p.m.; that of the tetrahydrocortisone eluate, more than 600 cpm and those of the plasma eluates from 50 cpm for the first to about 10 cpm for the last. It may be argued that the count rates of the plasma samples in relation to the background are questionable. An experiment was carried out to test the validity of this argument and the results are recorded /

recorded on Table 4. 4ml of a standard solution having a fairly high count rate were counted. Subsequently aliquots of 2ml, 1ml, 0.5ml, 0.3ml, 0.2ml, 0.1ml were counted and the observed count rates were compared with the theoretically true count rates (based on the count rate of the first sample). The data obtained suggested that a signal count rate of at least 10 cpm may still be fairly accurately counted.

Counting Efficiency of Small count rate

Table 4

<u>Std.Solution ml.</u>	<u>Observed c.p.m.</u>	<u>Theoretical c.p.m.</u>	<u>% Efficiency</u>
4	488	(488)	-
2	237	244	97
1	124	122	102
0.5	60	61	98
0.3	37	36	103
0.2	24	24	100
0.1	14	12	117

Washing and Disposal.

Particularly when low count rates are encountered, all glassware have to be scrupulously clean. This degree of cleanliness was achieved by washing all test tubes, flasks, bottles etc. with teepol using a brush, copious amounts of tap water and finally rinsing the glassware with distilled water. Pipettes were cleaned by dipping them into containers containing (a) solution of teepol (b) tap water (c) distilled water (d) acetone and flushed by suction. Disposal of radioactive 'waste' did not present a problem since ^{14}C is a /

a very weak β -emitter and the amount normally used was very small. An assurance had been obtained from the Medical Physics department of Edinburgh that washing the radioactive waste down the sink with plenty of water was quite safe from the point of view of public health.

Chromatography.

A large number of steroids present in blood and urine can effectively be separated into groups or individual components by chromatographic techniques. Several different chromatographic solvent systems are available for use with adrenocortical steroids and the choice must depend upon the specific requirements of the problem under study. Generally speaking, fractionation of the steroids are brought about by adsorption chromatography or/and partition chromatography.

In adsorption chromatography, a mixture of steroids in solution is passed through a finely divided adsorbent. The various steroids are adsorbed at different distances along the length of the column and may be separated either by cutting the column and dissolving each steroid in a suitable solvent or by passing a solvent through so that the various steroids pass out of the bottom of the column in an order which is dependent upon their physical properties and the adsorption of the column.

In partition chromatography, the column is impregnated with one solvent (the stationary phase) and the mixture of steroids dissolved in a second solvent (the mobile phase) passed through it. The steroids are separated in an order depending, not upon the adsorption, but upon their relative solubilities in the two solvents. This principle is applied in paper chromatography where filter paper is used as the support for the stationary phase when non volatile /

non volatile solvents are used; or the filter paper is allowed to become saturated with respect to the two solvents when volatile solvents are used. Paper chromatography has some advantages over column methods - several samples may be analysed at the same time.

Two paper chromatographic procedures had been used in the measurement of hydrocortisone production rate. When blood was the starting material, isolation of plasma hydrocortisone was the factor which determined the use of the Bush⁵ (1952) solvent system. Volatile solvents were used in this partition system which was composed of aqueous methanol as the stationary phase and benzene saturated with methanol as the mobile phase. When urine was the starting material, isolation of tetrahydrocortisone the main metabolite of hydrocortisone, was the main factor which determined the use of the Zaffaroni (1953) solvent system subsequently modified by Nowaczynski and Koiw (1957), in that ethylene glycol/toluene as the partition system was substituted for Zaffaroni's original propylene glycol/toluene system. This modification resulted in cutting down the running time from 96 hours to 16 hours without loss of sharpness of separation.

Paper.

With the Bush⁵ system Whatman No.1 paper was used and with the Zaffaroni system, Whatman No.54. In both cases, the paper was cleaned by exhaustive capillary washing continuously for 3 days with 95% methanol to reduce the 'blank'. Also in both cases, descending chromatography was carried out in the dark and at room temperature. The paper was cut into strips, 45cm long and 14cm wide and further subdivided to give five 2cm limbs for use with the benzene-methanol-water system and two 4cm limbs with the ethylene glycol/toluene system. The starting line was ruled 12cm from the upper edge and the limbs /

limbs were joined at the top and the bottom to prevent tangling and cross contamination during handling.

"Spotting-on" Support.

It is often difficult to find a convenient support for the paper when applying the spots of material on to it. The apparatus used and to be described here kept the chromatography paper clean and also held it in such a way that a drying current of air could be continuously applied from above or below. It consisted of two rectangular plates 50cm x 28cm in plywood joined at one edge by adhesive plaster so that both plates could be opened or closed like the covers of a book. A sector 20cm x 3cm was cut out of each plate in the same position so that the paper to be spotted on was sandwiched between the plates and only that part of the paper to be applied was exposed. At the time of application, the plates were placed on a support at one end with that part to be applied slightly in a raised position. This arrangement not only facilitated operation but also allowed the drying current of air from a hair dryer to be channeled upwards directly on to the point of application thus limiting the diameter of the spot to a minimum.

In both procedures, rectangular Shandon all glass chromatographic tanks, 57cm x 30cm x 20cm were used. The tanks were lined on all sides by paper always kept wet with the mobile solvent present at the bottom of the tank to a depth of about $\frac{1}{2}$ ". In the Bush₅ tank, a 500 cc beaker, also lined with paper but containing the stationary solvent was placed on the floor of the tank - this solution was changed every fortnight. The covers of both tanks were cushioned with foam rubber and soft transparent plastic sheets. This arrangement enabled the covers, by means of rubber bands, to sit more /

more tightly on the tanks, thus efficiently keeping the atmosphere in the tank saturated with the vapours of the solvents. In the modified Zaffaroni system where the paper had first to be impregnated with the stationary phase, ethylene glycol/methanol (1:1), equilibration was not necessary. With the Bush₅, equilibration was allowed to proceed overnight (for convenience). The mobile phase was introduced through a stoppered hole in the middle of the cover and lead directly into the trough.

Location, Elution and Quantitation.

To locate the position of the plasma hydrocortisone spots, the dried paper was held against an ultraviolet lamp and the positions of the hydrocortisone markers, recognised as opaque spots, were marked out. Zones on the other limbs, directly opposite these markers were cut out. Very rarely did the amount of hydrocortisone present in 10ml plasma show up as spots to the naked eye. The position of the urinary tetrahydrocortisone was determined by developing one of the limbs with blue tetrazolium solution made up of 1 part of 0.5% BTZ in water and 3 parts of 10% NaOH in 60% aqueous methanol. As mentioned earlier, two distinct mauve bands appeared within a few seconds; the one nearer the origin was due to tetrahydrocortisol and the broader one further away from the origin was due to tetrahydrocortisone. A zone opposite to this broader band was cut out from the other limb and in all cases elution of the steroids were carried out by flutting the cut paper and immersing in ethanol overnight.

Quantitation of the tetrahydrocortisone was carried out by reaction with Blue tetrazolium made alkaline with tetramethylammonium hydroxide. Maximum colour development was allowed to proceed at room temperature, in the dark and at the end of 90 minutes the /

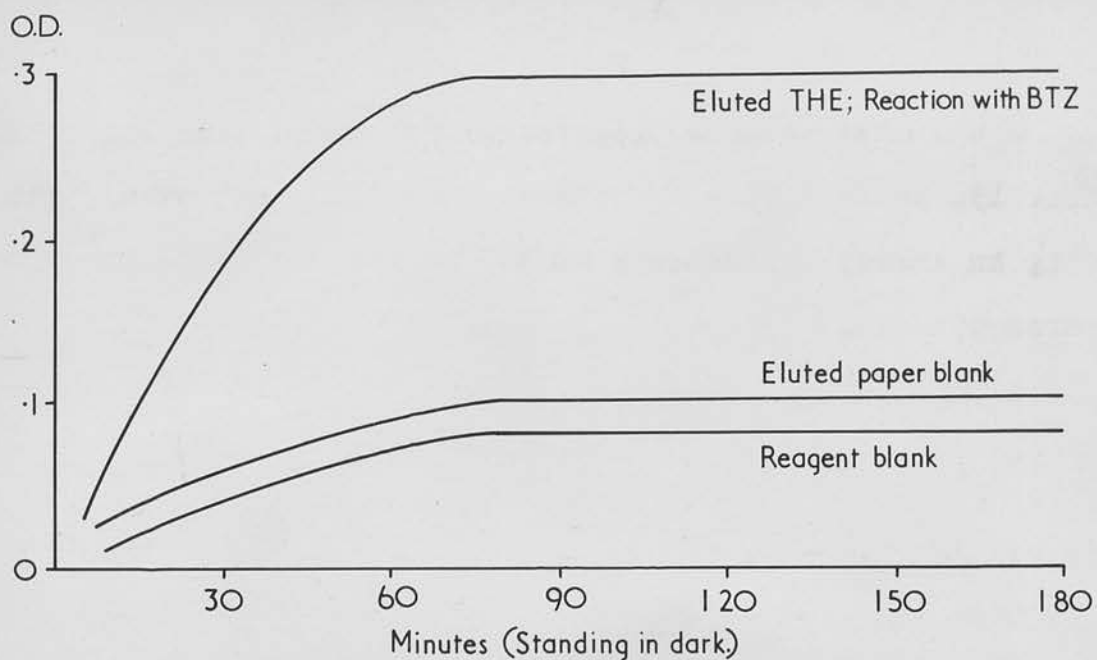


Figure 12: Development and stability of colour VS Time.

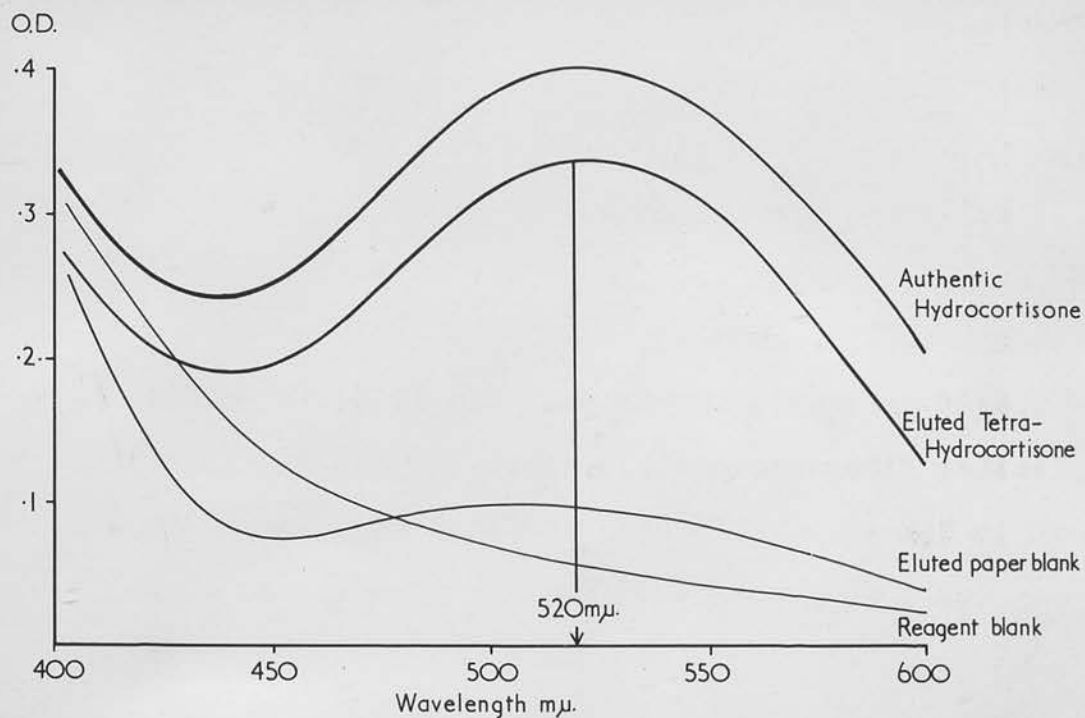


Figure 13: BTZ reaction absorption spectra.

the optical density was read at a wavelength of 520 mμ. Figure 12 shows a plot of colour development against time, indicating that standing in the dark at room temperature for 90 minutes was sufficient and Figure 13, shows a plot of optical density versus wavelength indicating an absorption maximum at 520 mμ for authentic and eluted tetrahydrocortisone.

The quantitation of plasma hydrocortisone was carried out by fluorimetry and this will now be discussed.

Fluorescence of Steroids.

It has been known for many years that various steroids when treated with certain concentrated or anhydrous acids, fluoresce brightly under suitable illumination. Wieland, Straub and Dorfmueller (1929) found that oestrogenic hormones developed a yellowish orange colour and a bright green fluorescence when dissolved in a mixture of concentrated sulphuric acid and acetic anhydride. In 1936, Wintersteiner and Pfiffer reported a similar fluorescence when cortisone and hydrocortisone were treated with concentrated sulphuric acid. In 1951, interest in this phenomenon was revived by the finding of Neher and Wettstein that all the physiologically active steroids when isolated by paper chromatography fluoresced with distinctive colours under ultraviolet illumination and when heated with phosphoric or sulphuric acid or acetic anhydride. In 1952, Finkelstein developed a quantitative fluorophotometric assay for certain corticosteroids dissolved in 85% phosphoric acid and with the aid of heat. In 1954, Sweat reported a more sensitive method in which the steroids were dissolved in concentrated sulphuric acid at room temperature. In this research, this method described by Sweat was largely employed. It was simple and very sensitive. With suitable fluorometers, a /

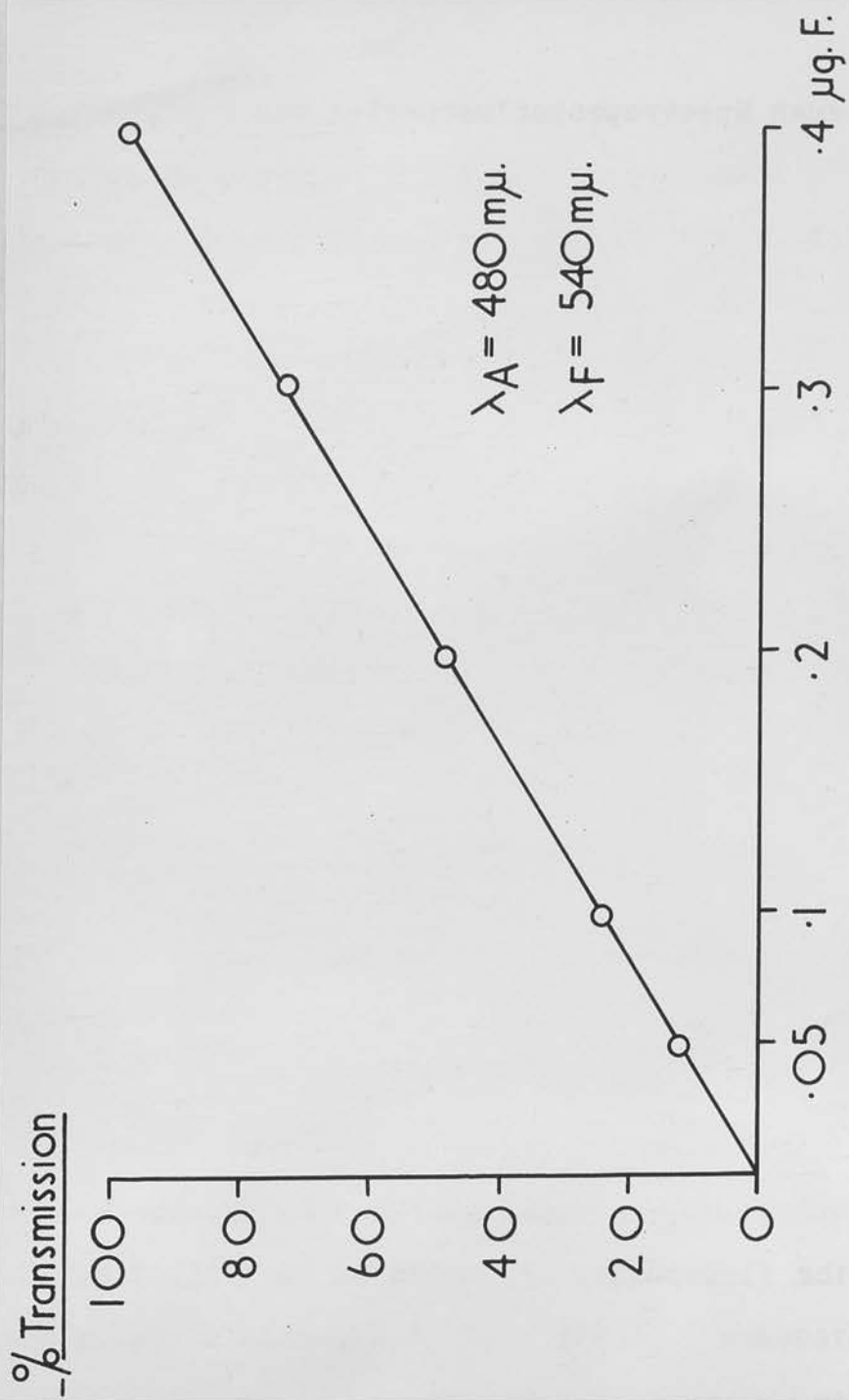


Figure 14: Relationship between fluorescence and concentration of Hydrocortisone.

a quantity as little as 0.05 μ g of hydrocortisone may still be measured.

Apparatus.

The Aminco-Bowman Spectrophotofluorometer was used. The assembly consisted of a Xenon Lamp Ballast and Control, an optical unit, a photomultiplier, a cathode-ray oscillograph and a recording drum. Light from the Xenon lamp is dispersed by the activating monochromator (grating type) and the monochromatic radiation falls on the sample. A similar monochromator disperses the fluorescent light from the sample into a monochromatic radiation and this falls on a photomultiplier tube. The light is transformed into a weak electrical signal and fed into a photometer which is amplified. The photometric output may be recorded from the tracings on the recording drum or from a scaler unit in terms of percentage transmission or optical density.

Experimental.

0.5ml (or 1ml) of the eluate was evaporated to dryness and the residue redissolved in 0.2ml alcohol. 1.8ml of 80% H_2SO_4 were rapidly added to it - the contents being well mixed - and left to stand in the dark at room temperature for 40 minutes. The acid mixture was then transferred to a fused quartz cell and the fluorescence measured in the fluorometer operating at an activating of 480 m μ and a fluorescent of 540 m μ . A standard of 0.1 μ g or 0.2 μ g of hydrocortisone treated likewise was read with the unknown sample. The hydrocortisone standard was read against an alcohol blank and the plasma hydrocortisone samples against paper blanks - the blanks being similarly treated as the standard or plasma hydrocortisone.

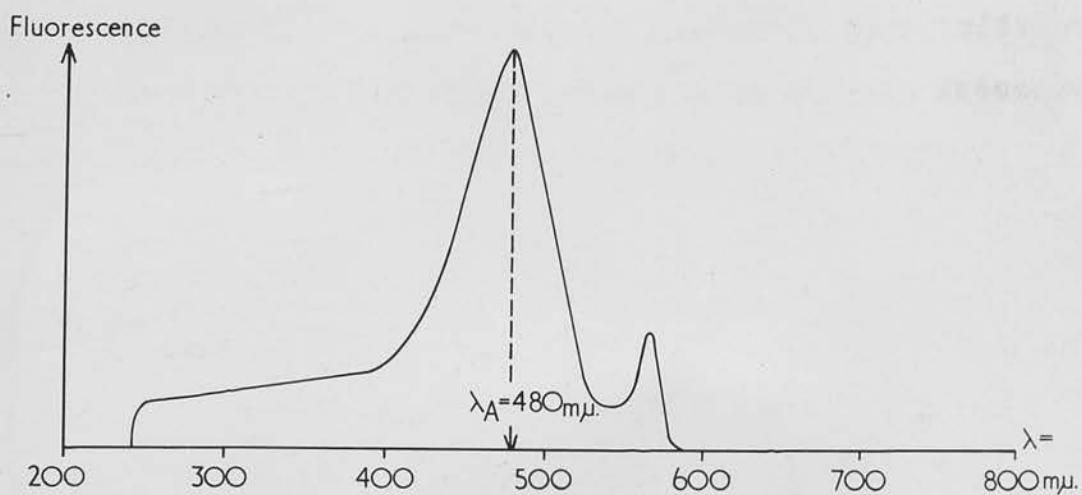


Figure 15(a): Activating Spectrum of Hydrocortisone.

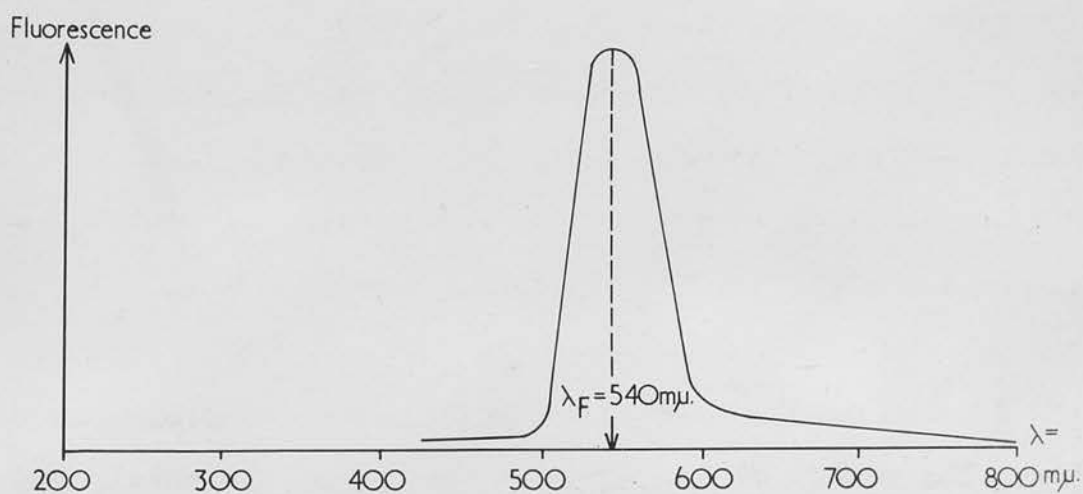


Figure 15(b): Fluorescent Spectrum of Hydrocortisone.

A linear relationship was found to exist between the intensity of fluorescence and the concentration of hydrocortisone over a wide range and Figure 14 shows this relationship from 0.1 μ g to 0.4 μ g hydrocortisone - a range which corresponds to 10 μ g% to 40 μ g% of hydrocortisone in plasma. Figures 15a, and b, show the activation and fluorescent spectra of authentic 0.2 μ g hydrocortisone. A maximum activating absorption was obtained at 480 m μ and a maximum fluorescence absorption at 540 m μ .

Fluorescence takes time to develop and Figure 16 shows the relationship of development and stability of fluorescence with respect to time. The graph shows that maximum fluorescence developed about 20 minutes after the addition of the acid to the alcoholic steroid solution. The fluorescence remained steady for at least 2 hours after which the intensity of fluorescence diminished progressively.

The value of the fluorometric method of measurement of hydrocortisone and of other steroids lies in its simplicity and marked sensitivity. Although claims have been made that acid induced fluorescence of certain steroids are specific reactions, it is generally agreed from the results of many studies, that the chromogenic and fluorogenic properties of steroids treated with concentrated acids result from very complex and relatively unspecific reactions. The course of these reactions can be profoundly influenced by relatively small changes in time and temperature of reaction exposure to light, amount of trace contaminants and other reaction conditions. Fortunately too because of this, a wide variety of reaction procedures can be selected, each of which favours a specific steroid or class of steroids.

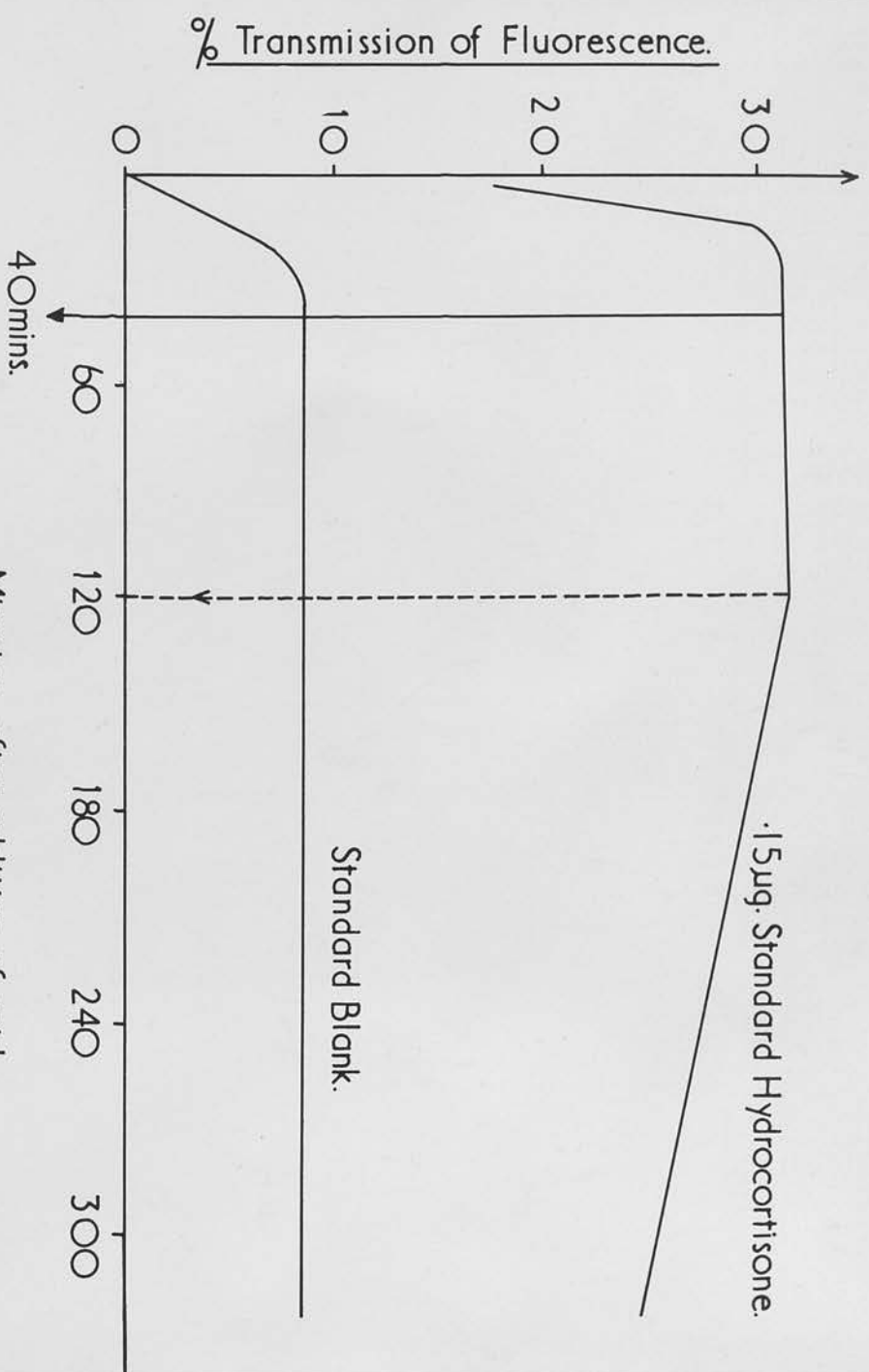


Figure 1b. Development and stability of Fluorescence of Hydrocortisone.

$\lambda_A = 480m\mu.$
 $\lambda_F = 540m\mu.$

Miscellaneous.

It is of interest to point out once again, that the great advantage of using radioactive isotopes in the measurement of hydrocortisone production rate is the overcoming of the problems of losses due to hydrolysis, extraction, washing, transfer to paper etc. In the two methods previously described the calculated production rates by isotope dilution depended upon the evaluation of the specific activities (either of hydrocortisone or of tetrahydrocortisone) and these were not affected by incomplete recoveries. Still, it was felt desirable to assess such losses - as there were bound to be - particularly in connection with the measurement of plasma hydrocortisone levels.

In a series of eight plasma samples withdrawn at 9.30 A.M. from patients with no apparent endocrine abnormalities, 10ml of the plasma in each case were subjected to the whole process of extraction, washing, chromatographic isolation, elution and fluorometric determination. The observed (uncorrected for losses) levels of plasma hydrocortisone are shown on Table 5. A normal range of 4.0 - 8.8 $\mu\text{g}\%$ and mean of $6.6 \pm 1.5\mu\text{g}\%$ were obtained.

When known amounts of authentic hydrocortisone were added to the plasma, the percentage recoveries obtained are presented on Table 6. A range of 52 - 65% of the added hydrocortisone was removed with a mean recovery of $59 \pm 4\%$. Although there appeared to be good reproducibility in the method used, the loss due to the various steps was considerable.

Table 5 Eight Normal Plasma Levels of F

<u>Case</u>	<u>Level</u>	
No. 1	4.0 ug%	in duplicate (mean)
2	5.6 "	"
3	7.4 "	"
4	8.8 "	single determination
5	4.7 "	"
6	6.9 "	"
7	6.7 "	"
8	8.2 "	in duplicate
MEAN	6.6 \pm 1.5ug%	

Table 6 % recovery of added F to Plasma

<u>Added to 10 ml Plasma</u>	<u>Recovery</u>
1 ug	55 %
1 ug	55 "
1 ug	53 "
2 "	52 "
2 "	65 "
2 "	63 "
3 "	55 "
3 "	60 "
	MEAN 57 \pm 4 %

The next experiment was designed to find out if this loss could be due to that stage of the analysis which involved transfer of the extract on to the paper and to chromatography, elution and fluorimetric estimation. Accordingly, aliquots of standard hydrocortisone corresponding to 1, 2, and 3 ug were pipetted into glass stoppered test tubes and evaporated to dryness. The residues were redissolved in small quantities of methylene chloride and transferred to Whatman paper, chromatographed, eluted and subjected to fluorimetric estimation. The results of this experiment are recorded on Table 7. A mean recovery of $99 \pm 9\%$ was obtained implying that virtually no loss was due to this stage of the analysis and that the loss reported earlier must have come from the extraction and washing stages.

Table 7 % Recovery from Paper Chromatography.

<u>Applied to Paper</u>	<u>% Recovery</u>
1 ug	117, 104, 112, 97 (in quadruplicate)
2 "	92, 92, 85, 92 "
3 "	101, 103, 96, 94 "
	MEAN $99 \pm 9\%$

To test this implication, known amounts of radioactive hydrocortisone were added to plasma and the plasma extracted with 100 ml methylene chloride. The extracts were washed once with 15 ml N/10 NaOH, once with 15 ml N/10 CH₃COOH and once with 15 ml distilled H₂O. They were then evaporated to dryness and the residues, redissolved in ethanol were counted for recovered radioactivity. The results of this experiment are recorded on Table 8.

Table 8 % Recovery from Extraction and Washing

<u>Specimen</u>	<u>Radioactivity added to 10ml Plasma</u>	<u>Counts recovered</u>	<u>% Recovered</u>
1	3570 c.p.m.	2292 c.p.m.	64 %
2	3570 "	2295 "	64 %
3	3570 "	2314 "	65 %
4	3570 "	2078 "	58 "
MEAN			63 \pm 3 %

From the four dererminations, a recovery of $63 \pm 3\%$ was obtained. This conclusively indicated that the overall loss of about 40% was due mainly to the extraction and washing stages.

The urine method, as was mentioned earlier, depended upon the measurement of the total count (Cu) and the specific activity of the isolated tetrahydrocortisone (SA_{THE}). The measurement of this latter presented no problem but that of the former did, since the incorporation of 0.5 ml urine into the liquid phosphor produced a slight turbidity and this would tend to depress the true count rate of radioactivity present. Correction for this quenching was brought about by the employment of an internal standard of known count rate which was of the same order as the activity found in the urine. To test the validity of this operation, 4 different 0.5 ml urine samples were used. To each, a quantity of radioactivity equivalent to 103 c.p.m. and an internal standard were added. Observations were made before and after the addition of the internal standard and the results are presented on Table 9.

30

Table 9 Validity for Correcting for Quenching.

<u>Urine Sample</u>	<u>Added</u>	<u>Observed</u>	<u>Corrected</u>	<u>% Accuracy.</u>
1	103 c.p.m.	71 c.p.m.	104 c.p.m.	101 %
2	103 "	90 "	98 "	95 "
3	103 "	89 "	106 "	103 "
4	103 "	94 "	104 "	101 "
				MEAN 100 \pm 3%

The results show that the employment of an internal standard to correct for quenching effects was essential and the method used was quite reliable.

In summary it may be concluded from the foregoing observations that a) the levels of plasma hydrocortisone in 'normal' individuals, as estimated by the blood method described, range from 4.0 - 8.8 ug % with a mean of 6.6 ± 1.5 ug %, and that these uncorrected values represent about 60% of the true value; the loss of the remaining 40% being mainly due to the extraction and washing stages in the method. b) Correction for quenching due to urine is essential and valid since the method employed recorded an accuracy of 100 ± 3 %.

Chapter III : Results and Discussion

Results.

When the methods to be used became established, it seemed desirable first of all to investigate the normal production rate patterns (using both the urine and blood methods) before actually applying them to the investigation of disease states. Accordingly, from a series of seven healthy young adults between the ages of 22 and 31 years, a normal range was obtained. Each was given a dose containing about 0.1 μ c to drink and 24 hour urine was collected. The results of this first investigation are summarised on Table 10.

Table 10 Normal F Production Rate (Urine Studies)

Case	Age	Sex	Dose Administered c.p.m.	Excreted/ 24 hr (Cu) c.p.m.	% Excreted	Specific Activity THE	F Production Rate mg/24hr.
G.d.W.	31	M	130000	114060	88	10.1	11.4
A.d.W.	24	F	130000	123200	95	14.0	8.8
M.S.	26	M	130000	101800	83	9.1	12.0
T.I.	24	M	130000	108000	83	5.7	18.9
H.M.	28	M	130000	100940	78	8.5	11.8
J.B.	26	M	130000	110960	85	7.5	14.7
I.W.	22	M	130000	107200	82	6.2	17.3
					83 \pm 5		13.3 \pm 3

A normal range of 8.8 - 18.9 mg/24hr with a mean of 13.3 \pm 3 mg/24hr was obtained. This compared well with Cope and Black's normal range of 5 - 24 mg/24hr with a mean of 11.8 \pm 2.25 mg/24hr which was obtained from investigating a series of eight convalescent subjects, presumably from a wider age group. The results also showed that excretion of the administered dose was almost complete in 24 hours and the amount ranged between 78 and 95% with a mean of 83 \pm 5%. Data /

Data have also been obtained in the course of one special investigation which suggested that during the first 8 hours following the administration of the radioactive material, 50% of the dose was excreted into the urine. During the subsequent 8 hours some 20% and during the last 8 hours another 10% giving a total urinary excretion of about 80% in 24 hours.

A study of the distribution of the urinary ^{14}C seemed worthwhile investigating; for example it would be of interest to know what fraction of the administered dose appeared in the urine as free unconjugated steroids, or as β -glucuronidase hydrolysable steroids or as tetrahydrocortisol (THF) and as tetrahydrocortisone (THE). In order to evaluate these fractions, one aliquot of 50ml urine was acidified to pH 1 and extracted with an equal volume of chloroform. The extract was then washed with 10ml N/10 NaOH, with 10ml distilled water and evaporated to dryness. The residue was redissolved in alcohol and its activity measured. This fraction was taken to represent the free unconjugated urinary steroids and the activity obtained was expressed as a percentage of the dose administered. A second 50ml aliquot was brought to pH 4.6 and subjected to β -glucuronidase hydrolysis at 37° for 24 hours. After which, the urine was extracted with chloroform, washed and dried. The extract was redissolved in alcohol and counted for radioactivity. This fraction was taken to represent the conjugated, β -glucuronidase hydrolysable steroids. A third aliquot was brought to pH 4.6, subjected to enzymatic hydrolysis, extracted and chromatographed. The tetrahydrocortisol and tetrahydrocortisone zones were located, cut and eluted overnight. The activities of these two zones were then counted and from a series of 4 cases investigated, the results are presented on Table 11.

Table 11 Distribution of ^{14}C in 24 hour Urine.

Case	Dose Admin- istered c.p.m.	% of Dose Excreted in 24hrs	% of Dose in pH1 extract	% of Dose in glucur- onidase extract	% of Dose in THF zone	% of Dose in THE zone
T.I.	130000	83	0.98	21	1.0	5
H.M.	130000	78	0.42	18	1.1	7
J.B.	130000	85	0.97	16	1.3	4
I.W.	130000	82	0.62	24	1.3	7

The results obtained suggested that less than 1% of the administered dose existed in the urine as free unconjugated steroids; between 16 and 24% as β -glucuronidase hydrolysable steroids; between 1 and 1.3% as isolated tetrahydrocortisol and between 4 and 7% as tetrahydrocortisone. From consideration of the total amount excreted in the urine and the amount in these various fractions, it will be noted that a large percentage is 'hydrophilic' and not extractable by chloroform.

As an extension to the above studies, the quantitation of tetrahydrocortisol and tetrahydrocortisone together with the measurement of their specific activities had also been carried out and the results are summarised on Table 12. The results obtained suggested a normal excretion of 297-560 $\mu\text{g}/24$ hr. for tetrahydrocortisol and 941-2832 $\mu\text{g}/24$ hr for tetrahydrocortisone. There also appeared to be a good correlation between the specific activities of the THF and THE fractions suggesting that the administered radioactive hydrocortisone had been treated by the body as if it were the endogenously produced hydrocortisone. It therefore entered in the same /

same proportion into all the metabolites imparting to them the same specific activity.

Table 12 Quantitation of THF and THE and measurement of their Specific Activities.

Case	TETRAHYDROCORTISOL		TETRAHYDROCORTISONE	
	µg/24hr.	SP. Activity	µg/24hr.	SP. Activity
T.1.	578	5.0	2177	5.8
H.M.	297	8.0	940	8.5
J.B.	397	8.5	1655	7.5
I.W.	560	5.8	2832	6.2
McC.	552	1.9	2016	2.2

When it is considered that to measure the hydrocortisone production rate from blood studies, the dose has to be injected and 25ml of blood have to be withdrawn every $\frac{1}{2}$ hour for $2\frac{1}{2}$ hours, it is not surprising that healthy 'normal' volunteers were very difficult to find. It was therefore suggested that convalescent subjects or patients with no apparent endocrine abnormality might be the next best thing to investigate and to use them as normals. Accordingly 3 convalescent subjects and 2 pre-surgical patients were subjected to the test and the results are summarised on Table 13.

The results obtained suggested that the normal hydrocortisone production rate, as measured from blood studies, ranged from 16.1 to 30.2 mg/day. Other information obtained indicated that the size of the miscible pool ranged from 0.87 mg to 3.1 mg; the biological half life from about 60 minutes to 120 minutes and a rate of replacement or turnover rate of 0.34 to 0.86 pool/hour. All these results agreed well with those obtained by Peterson and Wyngaarden, the /

Table 13 'Normal' F Production Rate (Blood Studies)

Case	Age	Sex	a μg	Ii cpm/ ug	Io cpm/ μg	t _{1/2} hr	A mg	K pool/ hr	KA mg/hr	KAx24 mg/day
J.T.	35	M	9.6	21750	120	0.95	1.73	0.73	1.26	30.2
G.E.	66	M	9.0	21950	64	1.90	3.10	0.39	1.11	26.6
W.	30	M	9.6	21750	130	1.65	1.60	0.42	0.67	16.1
A.W.	60	M	11.32	22497	290	0.83	0.87	0.86	0.75	18.0
H.C.	41	F	9.6	21750	215	0.98	0.96	0.71	0.68	16.3

$$A = \text{miscible pool} = a \left[\frac{I_i}{I_o} - 1 \right]$$

where a = amount of F in the dose

Ii = specific activity of dose

I_o = specific activity at zero time

$$K = \text{rate of replacement or turnover} = \frac{0.693}{t_{1/2}}$$

where t_{1/2} = half life expressed in hours

KA = Turnover per hour

KAx24 = Turnover per day

the original authors of this method. These values furthermore, were somewhat higher than those obtained by the urine method. This must necessarily be so since this method assumed a uniform rate of turnover based on that measured between 9.30 AM and /

and 12.00 noon - a period of increased adrenal activity relative to the rest of the day; whereas the urine method measured a production rate which took into consideration all the variations attendant in the adrenal cortex during the 24 hour period of study.

The plasma levels of this series of 5 normals investigated during the period of experiment are presented on Table 14.

Table 14 Plasma F levels ($\mu\text{g}\%$) from 10.00AM-12.00 Noon

Case	10.00 AM	10.30	11.00	11.30	12.00 Noon
J.T.	7.7	5.8	7.3	7.7	6.5
G.E.	8.7	6.6	6.1	5.0	5.8
W.	6.7	3.6	3.1	3.6	3.6
A.W.	5.0	4.6	6.4	6.4	3.2
H.C.	5.0	5.0	5.6	5.6	3.1

The plasma levels of the first specimen taken at 10.00 AM, in all cases, agreed very well with the normal levels presented on Table 5. The interpretation of the levels in relation to time was difficult, simply because the patterns in all cases were irregular. If however only the 10.00 AM and the 12.00 Noon levels were considered, then it could be said that in all cases, the levels at 10.00 AM were higher, indicating to some extent the existence of a diurnal variation and the state of the adrenal gland during the forenoon and noon periods.

With the establishment of the normal production rate patterns, /

patterns, the stage was then set to apply these methods to the investigation of cases in which the adrenal glands were implicated. A number of Cushing's, Hirsute, Hypoadrenalism and miscellaneous cases were investigated using either one or both these methods. An attempt will therefore be made to present the results grouped in allied disease states.

A series of 13 patients, admitted for further investigation as possible cases of Cushing's disease were subjected to the isotope dilution test and the results, summarized on Table 15, are divided into two sections, one illustrating those which showed evidence of increased production rates and the other where normal values were encountered. Also shown on the table will be the results of the urinary 17-ketosteroids and 17-ketogenic steroids carried out simultaneously with the estimates of production rates so that a comparison might be made of the sensitivities of the various tests for adrenal function. Abnormal values in each case were underlined.

Eight of the 13 cases investigated registered a moderate to a markedly elevated production rate and five showed a normal production rate pattern. Of these eight cases with elevated values, cases 1, 2, 3 and 5 have since been treated by adrenalectomy. The rest, together with those who showed a normal production rate are still on 'observation' and on a restricted diet regime to reduce the obesity.

Only in 4 cases was the blood method applied and this test was discontinued when it was felt that the urine method, which was much simpler, was capable of giving just as valuable information. Although in most cases, the 17-ketogenic steroid excretion values indicated increased adrenal activity, the degree of sensitivity was

was poor when compared with either of the production rate methods. Only in two of the eight established cases did the 17-ketosteroid excretion register a raised level and this, furthermore, was only of a moderate degree. From all these results it does appear therefore that where the necessary facilities are available, the production rate method by isotope dilution appears to be the method of choice simply because it is a more sensitive index of adrenal function than either the 17-KGS, which is its next rival, or the 17-KS estimation.

Table 15 F Prod. Rates of 13 cases investigated as possible Cushing's

Case	Age	Sex	F PRODUCTION RATE		17-KS mg/day	17-KGS mg/day
			BLOOD mg/day	URINE mg/day		
1. M.St.	34	F	<u>40.8</u>	<u>22.6</u>	12.9	<u>24.4</u>
2. M.Sr.	39	F	<u>70.0</u>	<u>112.0</u>	<u>16.9</u>	<u>32.6</u>
3. T.D.	38	M	<u>62.4</u>	<u>75.8</u>	19.4	<u>28.6</u>
4. F.	19	M	-	<u>51.6</u>	-	-
5. R.Mc.	39	M	-	<u>35.1</u>	<u>23.6</u>	5.6
6. S.	30	F	-	<u>33.0</u>	11.0	<u>24.7</u>
7. G.F.	26	M	-	<u>22.8</u>	13.2	<u>26.3</u>
8. W.	53	F	-	<u>25.9</u>	13.1	<u>17.1</u>
9. lsb.	52	M	-	17.4	13.8	15.5
10. S.D.	12	F	7.2	11.1	2.6	2.5
11. F.B.	12	F	-	17.7	-	-
12. R.	19	F	-	14.9	10.6	11.7
13. W.S.	21	F	-	17.2	13.6	<u>20.4</u>

The results from investigation of 4 cases suspected with hypoadrenalism are presented on Table 16.

Table 16 F Production Rate in 4 Hypoadrenal Cases

Case	Age	Sex	Diagnosis	F PRODUCTION RATE		17-KS mg/24hr	17-KGS mg/24hr
				BLOOD mg/24hr	URINE mg/24hr		
14.J.A.	34	F	HYPOPIT.	3.4	3.6	0.6	0.8
15.M.R.	56	F	Addison's	4.9	1.7	0.2	NIL
16.Mit.	48	F	Addison's	-	8.3	3.9	6.5
17.P.T.	19	M	HYPOGONAD.	13.2	17.8	13.5	13.5

The results of cases 14 and 15 fairly clearly showed a reduced production by all the four indices of adrenal function. That of case 16 was somewhat dubious but in the light of the case history and clinical examination this was thought indeed to be an early case of Addison's disease and subsequent stimulation tests confirmed the diagnosis. The results of case 17, at least showed that the patient's adrenals were functioning normally and the information obtained from ACTH stimulation, dexamethazone suppression and urinary gonadotrophins suggested that the hypogonadism was of a hypothalamic type and accordingly he was treated by an implantation of 600 mg of testosterone.

A series of 7 hirsute cases was also investigated by the blood and urine methods for F production. It is generally believed that in this clinical condition, the 17-KS excretion may be raised or may lie in the upper normal limit and the 17-KGS which reflect the corticosteroid output may be in the normal or lower normal region./

Table 17 F Production Rate in Seven cases of Hirsutism

Case	Age	Sex	F Production Rate		17-KS mg/24hr	17-KGS mg/24 hr
			BLOOD mg/24 hr	URINE mg/24 hr		
18,McI,	34	F	<u>12.2</u>	10.7	7.8	15.6
19,E.C,	23	F	<u>14.8</u>	12.3	11.8	12.0
20,M,D,	48	F	<u>11.8</u>	13.3	6.0	6.0
21,J.M,	23	F	<u>13.4</u>	11.2	9.9	7.7
22, R,	30	F	<u>9.4</u>	11.2	15.0	14.8
23,A,N,	28	F	18.8	13.8	5.9	6.0
24, S,	26	F	-	<u>26.6</u>	<u>17.3</u>	<u>22.9</u>

region. The big question is, is the adrenal gland in this clinical state 'just' hyperactive to cause elaboration of 'just excessive' androgenic steroids and - due to 11-hydroxylase deficiency - just normal or subnormal corticosteroid output; or is the gland hyperactive only in one respect, namely, in the production of androgens and normal in the secretion of corticosteroids? To answer this question (or part of it), 17-KS, 17-KGS and hydrocortisone production rate investigations were carried out and the results are summarised on Table 17.

The results show that six of the seven hirsute cases registered a normal hydrocortisone production by the urine method and excepting case 24 who had high values in all departments, the 17-KS and 17-KGS excretions in all cases were also within normal limits. On the basis of a normal production of 16-30 mg/day by the blood method, the corresponding values registered by five of the cases /

cases in this series appeared to be somewhat subnormal with a range of 9.4 - 14.8 mg/day. Only case 23 showed a normal production of 18.8 mg/day. Brooks and Prunty (1960), from a study of the excretion values of 17-oxosteroids in a large number of hirsute women, are of the opinion that adrenocortical steroid production is normal in this clinical condition.

Discussion

Excretion and distribution of ^{14}C

The fate of administered hydrocortisone, radioactive or otherwise, and its excretion rates in a variety of conditions have been studied by many workers. Hellman et al (1954) administered 0.8 - 1.0 μC of radioactive material containing 0.25-100 mg hydrocortisone to normal subjects and to an adrenalectomised subject, both with and without a maintenance dose of hydrocortisone. The amount of radioactivity excreted in all cases 3 days after the administration was of the order of 80%. These results suggest that the rate of metabolism is independent of tissue requirements and of the amount present in the body. Furthermore since at the end of the infusion, only 13% remained as unaltered hydrocortisone, the rate of metabolism may be inferred to be rapid. These workers also reported that excretion of the radioactive material is prompt; that during the first 6 hours after infusion, 50-60% of the administered dose will have been excreted in the urine and that at the end of 24 hours the excretion may be expected to range from 72-84%. Only 5% had been found in the faeces during the period 24-48 hours and at the end of 4 days only a small percentage could be detected in the acetone extract of the faeces. The very small amount of radioactivity ($< .5\%$) reported to be present as CO_2 in expired air indicated that /

that degradation of ring A represented only a minor pathway.

Peterson et al (1955) reported that oral absorption is rapid and complete from the study of peak levels after oral and intravenous administration of 1-2.5 μ c of radioactive hydrocortisone and the similarities of recoveries and rates of excretion of metabolites. From the rates of diminishing specific activities in 10 normal subjects, they reported a biological half-life of 60-90 minutes and from urine studies a half-life of 3.8-4.8 hours. Also in this report, these workers confirmed Hellman's findings that infused hydrocortisone was metabolised at a constant rate whatever the dose given; but in patients with cirrhosis of the liver, the half-life of infused hydrocortisone was observed to be longer than that of normal subjects showing that liver damage affected steroid metabolism. Again it was reported that 70-82% of the dose were excreted in the urine during the first 24 hours or 86-94%, 72 hours after the administration of the radioactive material. Quite similar results were reported by Plager et al (1954) who having injected as much as 5 μ c in a 24 year old male subject, recovered some 3.7 μ c in the urine in 48 hours.

Migeon et al (1956) in a detailed study of the cumulative excretion pattern of radioactive material in urine reported that 2 hours after administration, 2-14% were recovered; in 4 hours 25-33%; in 8 hours 40-60%; in 14 hours 60-70%; in 24 hours 70-83%; in 48 hours 80-90% of the administered dose were found excreted in urine. Cope and Black (1958) reported an excretion range of 76-95% in 24 hours with a mean of $86 \pm 3\%$. In this thesis, the urinary excretion range of 78-95% with a mean of $83 \pm 5\%$, reported earlier, agrees well with all the previous findings. However, no attempt was made to study the excretion in faeces or whether in fact expired air contained small amounts of radioactive CO_2 as reported by Hellman et al.

Since the urinary excretion of the radioactive material is so rapid and the administered dose is small, being about 1 - 5 μC in America and 1.25 μC or less in the U.K., the danger to which the recipient is exposed to is small; more so if one considers that the maximum body burden for ^{14}C is some 200 μC .

Of the total ^{14}C excreted in the urine as illustrated on Table 11, the amounts present in the pH1 extract, in the glucuronidase hydrolysed extract and in the THF and THE zones of the chromatogram, were all smaller than those reported by Cope and Black (1958), Peterson et al (1955) and Migeon et al (1956). Less than 1% of the administered dose was found in the chloroform extract at pH1 which contained mainly the free cortisol and cortisone. From the same extract, Cope and Black reported a recovery of 1.5%, Peterson et al obtained 4% and Migeon et al 2%. A range of 16-24% of the administered dose was found in the chloroform extract made after β -glucuronidase hydrolysis. Against this value, Cope reported a recovery of 10-30% and Migeon obtained 29-40%. The nature and amount of extracting solvent used and the period of hydrolysis and extraction appear to account for these differences reported; a point which once again illustrates the advantages of this isotope dilution technique where a poor recovery will not affect the calculation of the true production rate.

Production Rate from Urine Studies.

When ^{14}C hydrocortisone is administered, as far as is known, it appears to be treated by the body in exactly the same manner as the endogenously produced hydrocortisone. It is therefore expected to be metabolised equally giving rise to the same proportion of the metabolic end products and to impart the same specific activities /

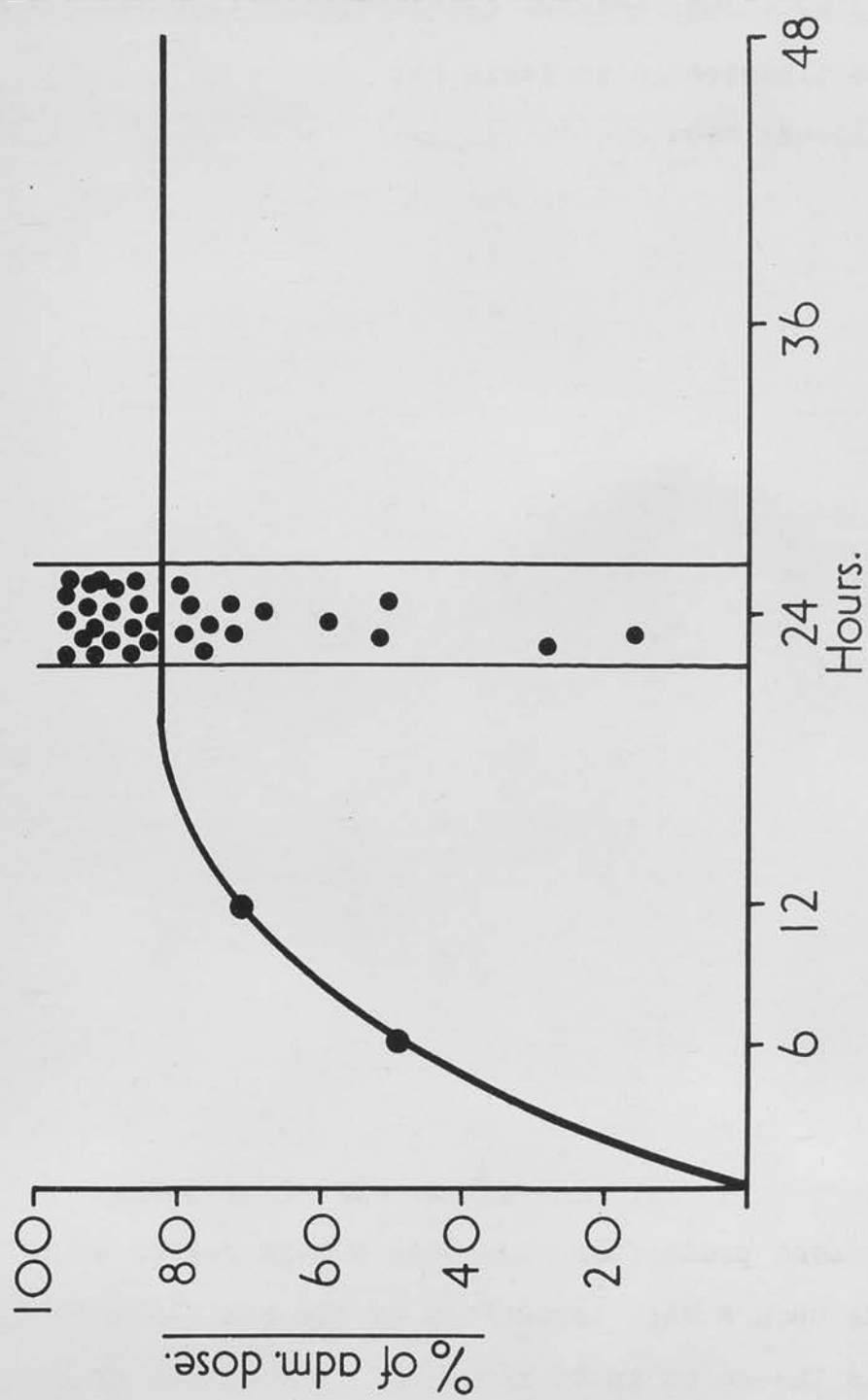


Figure 17: Cumulative excretion rate of C^{14} in urine.

activities to the various metabolites. Evidence that this is indeed the case is forthcoming from the studies of the specific activities of the abundantly available urinary tetrahydrocortisol and tetrahydrocortisone as illustrated on Table 12.

As has already been shown, the measurement of hydrocortisone production rate from urine studies depends upon a) the total activity in the 24 hour urine and b) the specific activity of the hydrocortisone metabolites. A variety of reasons may lead to a low excretion of ^{14}C and therefore affect the measurement of the true production rate. Losses may be due to incomplete administration, incomplete absorption from the alimentary tract, retention in the tissues or body fluids, impaired renal function and most frequently to incomplete collection, particularly if loss of urine occurs during the first few hours after administration. If however, the excretion in 24 hours is found to be 80% or more, then it would appear that these interfering factors play only a minor role and that the calculated production rate represents 80% or more of the true production rate. In this research, more than 90% of the cases investigated registered excretion rates of over 80%, see Figure 17. Barring renal impairment, an excretion rate of lower than 50% was interpreted as being due to incomplete collection and a repeat test undertaken a week later. The validity of the method to reflect the actual hydrocortisone production rate with a high degree of accuracy therefore depends upon a high percentage of the administered dose being excreted in the urine in 24 hours and upon the determination of the specific activity of the abundantly available urinary tetrahydrocortisone. It does not depend however, upon the isolated THE being pure; it is necessary only that the reducing steroid in the /

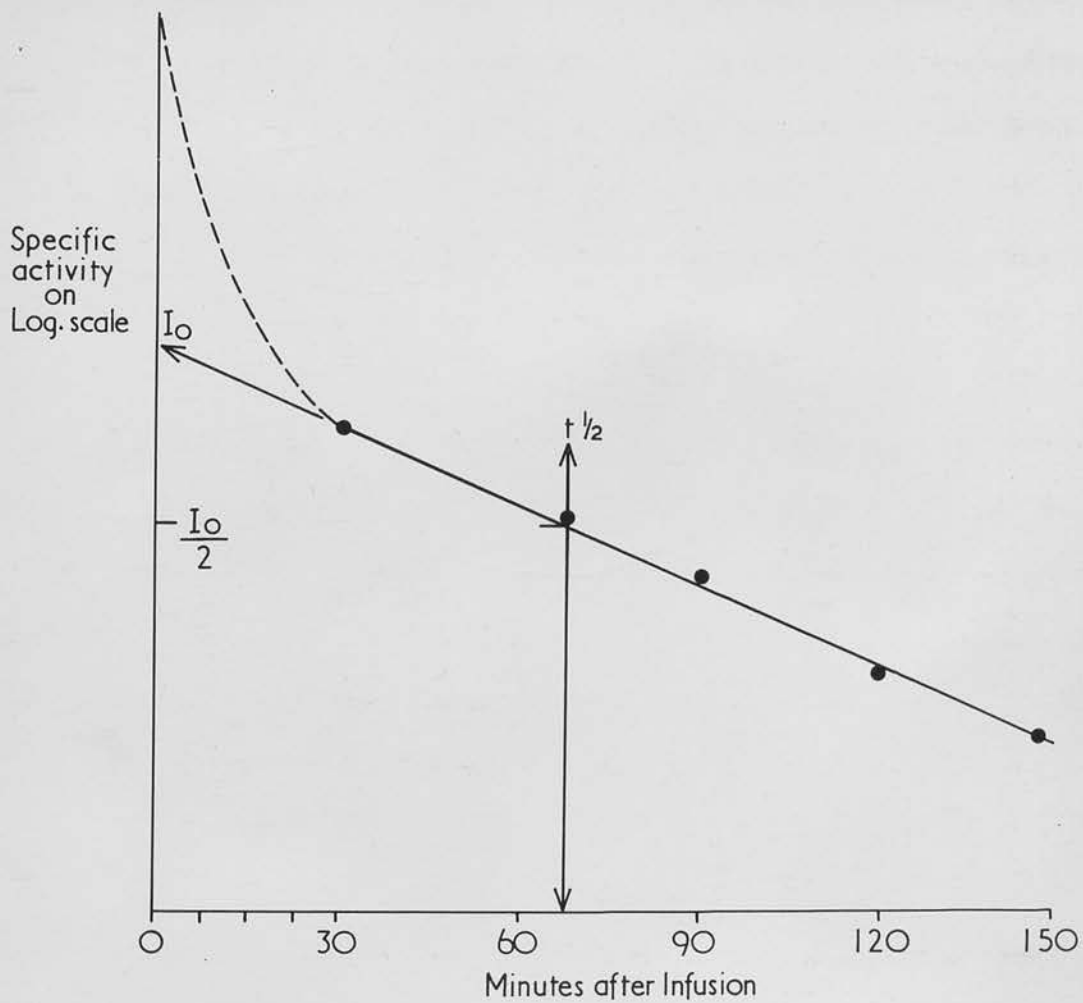


Figure 18: Plot of specific activities of Plasma vs time.

the THE zone of the chromatogram be derived solely from the metabolism of hydrocortisone.

Production Rate from Blood Studies.

The specific activities of the plasma hydrocortisone from the blood samples withdrawn 30, 60, 90, 120, 150 minutes after the administration of the radioactive material assume a straight line when plotted on a semilogarithmic graph; see Figure 18. The whole purpose of these serial determinations is to make possible the measurement of the specific activity at the time of complete mixing of the administered radioactive hydrocortisone with the non radioactive circulating hydrocortisone. This measurement may be obtained by extrapolating the line of fit to zero time. The ratio of the specific activity of the administered dose with that at the time of complete mixing ($\frac{I_1}{I_0}$) represents the degree of dilution of the administered amount of hydrocortisone (a) and from these two quantities the miscible pool (A) may be calculated from the isotope dilution expression :

$$A = a \left[\frac{I_1}{I_0} - 1 \right]$$

This rapidly exchangeable pool of hydrocortisone was defined by Peterson and Wyngaarden as that quantity of endogenously produced hydrocortisone in the body capable of mixing promptly and thoroughly with the intravenously injected labelled hydrocortisone. But since it is also known that hydrocortisone is rapidly metabolised in the body, this miscible pool, as determined, represents only a fair approximation of the actual very rapidly exchangeable pool of body hydrocortisone.

It has also been shown by Peterson and Wyngaarden (1956) that if the specific activities of plasma hydrocortisone are /

are determined at various intervals during the first 20 minutes after the administration of the dose, a rapid decline with respect to time may be observed as is illustrated by the dotted line on Figure 18. This very rapid initial decline, which is followed by a more gradual decline after 20 - 30 minutes, is attributed to the distribution of the labelled steroids in the exchangeable hydrocortisone pool. From consideration of the point where the first slope merges with the second, it may be inferred that equilibration is complete within 20 or 30 minutes after administration. The second more gradual declining specific activities which approximate a straight line on a semilogarithmic plot, results from the body's continual production of newly synthesized unlabelled hydrocortisone and the simultaneous removal of the steroid (labelled and unlabelled) from the pool. If it can be assumed that the size of the miscible pool is constant during the period of investigation, then the rate of turnover (K), which represents the amount of endogenously produced hydrocortisone added to the pool, may be calculated from the equation for the first order reaction :

$$I = I_0 e^{-Kt},$$

$$\text{from which} \quad K = \frac{\log 2}{t_{\frac{1}{2}}} = \frac{0.693}{t_{\frac{1}{2}}}$$

where I = the isotope content at any time

I_0 = " " " " zero time

$t_{\frac{1}{2}}$ = half time in hours (t = time in hours)

K = fraction of the pool replaced per hour.

As has already been mentioned, the product of the miscible pool (A) and the turnover rate (K) represents the turnover of hydrocortisone expressed as mg per hour. This quantity multiplied by 24 provides the turnover or production rate in mg/day.

In proposing this method, Peterson and Wyngaarden were cognisant of the various assumptions which have to be made and evaluated before it could be accepted as valid. Briefly their method assumes :

1. That the injected hydrocortisone is pure
2. That the injected hydrocortisone does not appreciably alter the size of the miscible pool and does not disturb the normal metabolism of the endogenously produced hydrocortisone.
3. That the size of the miscible pool remains constant throughout the period of study.
4. That the rate of synthesis of hydrocortisone equals the rate of transformation of the steroid
5. That the administered radioactive hydrocortisone is handled like the endogenous steroid, and that there is random disappearance of both the endogenous and labelled steroid.
6. That the mixing of the injected steroid within the pool is homogenous and is rapid compared with its metabolism
7. That the rate of metabolism of the steroid is proportional to its concentration
8. That the radioactive steroid does not re-enter the pool
9. That the isolated plasma hydrocortisone is pure.

In this research, a few of the assumptions have been tested while others have been inferred to be legitimate. The accompanying note on the ampoule containing the 4-¹⁴C cortisol, when it arrived from America, stated that it was 95% pure. This was checked by paper chromatography in the Bush system and it was found that 90-98% of the radioactivity moved as cortisol. This was taken to mean 100% purity and no correction was made in the calculation. The amount of hydrocortisone in the dose in every case ranged from 10-20 µg /

10-20 μg and this certainly would not alter appreciably the size of the pool or disturb the normal metabolism of the steroid. It has been suggested that the constancy of the size of the pool may, to some extent, be inferred from the constant plasma levels of hydrocortisone during the period of study. Data obtained from 25 investigations render this suggestion most difficult to accept partly because some patients show no significant variation in plasma F level between 10 AM and 12 noon while the majority of cases show a definite tendency to decline. Evidence that hydrocortisone once removed from the miscible pool does not re-enter the pool as unaltered hydrocortisone is forthcoming from the work of the original authors of the method. These workers, after the administration of a trace quantity of the labelled steroid, compared the specific activities of the circulating hydrocortisone which were sampled simultaneously from a peripheral vein and the hepatic vein by catheterization. The specific activities obtained from both sites were similar for at least three hours, indicating no apparent reversible concentration of hydrocortisone by the liver. Lastly data have been obtained in this research showing that the eluted steroid from the Bush⁵ chromatogram registered an identical absorption - fluorescent spectra with that of authentic hydrocortisone indicating that the isolated steroid is indeed pure hydrocortisone.

Cushings Syndrome.

Even when all the signs and symptoms associated with Cushing's syndrome are present, diagnosis still needs to be confirmed and the extent and nature of the adrenal hyperactivity evaluated by resort to biochemical investigations. When the diagnosis is established, the current treatment appears to be to subject the patient to adrenalectomy or to pituitary irradiation by ^{90}Yt . Apart from the /

the preliminary investigation for the presence of impaired glucose tolerance and electrolyte disturbance, the biochemical investigations usually carried out are the measurements of the urinary 17-KS, 17-KGS and where laboratory facilities permit, the estimations of urinary free cortisol, plasma cortisol and hydrocortisone production rate.

It has often been reported that the urinary 17-ketosteroids are moderately raised in Cushing's syndrome. In the 13 cases investigated (see Table 15) only 2 showed such an elevation while the rest registered a normal excretion pattern. Cope and Black (1959) investigating 12 cases of Cushing's disease, reported an insignificant rise of the mean value of 1.4 times the normal. Levell et al (1957), from 7 Cushing's cases investigated, reported a 17-KS excretion range of 4-40 mg/day. In fact only one of the 7 cases showed a raised value; the rest were well within the normal range. While normal excretion values of 17-KS do not exclude the possibility of Cushing's syndrome, high values, such as 100 mg/day or more as reported by Dorfman and Shipley (1956), not only confirm the diagnosis but often raise a strong suspicion that the cause of the hyperadrenalism is the existence of a tumour. The moderate increase in 17-KS in other instances is due mainly to the 11-oxygenated ketosteroids which are metabolites of cortisol and, to a lesser degree, to the 11-deoxy group which are metabolites of adrenal androgens.

From measurements of urinary 17-ketogenic steroids and 17-hydroxycorticosteroids, Cope and Black (1959) reported a 4-fold increase in Cushing's cases. Levell et al (1957) reported a range of 6-34 mg/day in 7 patients, four of whom showed elevated values. Six out of the thirteen cases reported on Table 15 registered higher than the normal excretion values for 17-KGS. Prunty (1960) reported /

reported that the level of 17-KGS in Cushing's syndrome is frequently found to be slightly elevated or within the upper normal limits and found this to be so in six out of his eighteen cases. He further suggested that excretion of these steroids is particularly liable to fluctuate from day to day in this syndrome and that if determinations are repeated, elevated values may be observed. For example, when nine determinations were carried out in one patient, values varied between 11 and 40 mg/day.

The measurement of the urinary excretion of free cortisol as a good diagnostic test for Cushing's syndrome has been proposed and supported by a number of workers. Cope and Black (1959), from a study of the cortisol output in hospitalized patients, observed a normal excretion of 0-100 $\mu\text{g/day}$ with a mean of 43 $\mu\text{g/day}$. In the 12 cases of Cushing's syndrome they investigated the excretion of this free steroid was raised some eight times the normal value giving a mean of 320 $\mu\text{g/day}$; only in 2 cases was there a small overlap with the normal range. Having also studied the 17-KS and 17-KGS excretion patterns simultaneously, they suggested that the estimation of urinary cortisol is at least twice as sensitive in detecting the hyperadrenalism of Cushing's syndrome as is the 17-KGS assay. Similar findings were reported by Ross (1960) who from a study of eleven patients observed excretion values up to 710 $\mu\text{g/day}$ as compared with his normal of 3-48 $\mu\text{g/day}$. Cope and Black suggested that the increased free cortisol found in the urine may be related to the change in the binding capacity of the cortisol to plasma proteins at different plasma concentrations. Mills (1959) found that the circulating cortisol was 95% bound at 37°C when the plasma concentration was 10 $\mu\text{g\%}$, 80% bound at a plasma concentration of 40 $\mu\text{g\%}$ and 72% bound when the plasma concentration was 80 $\mu\text{g\%}$. Thus in Cushing's /

Cushing's syndrome the binding capacity is reduced leaving a much greater proportion of the freely diffusable cortisol available for renal filtration.

On the basis of a normal plasma range of 4-8 $\mu\text{g}\%$ (60% recovery), the 10 A.M. plasma hydrocortisone levels from the first 3 Cushing's cases on Table 15 registered 13.1, 14.0, and 11.8 $\mu\text{g}\%$, thus providing some evidence that levels are moderately raised. Although high levels of plasma cortisol are expected in Cushing's syndrome - and indeed a level of 135 $\mu\text{g}\%$ was reported by Sweat (1955) - often, normal levels have been reported in many cases. Eik Nes et al (1955) have drawn attention to the fact that the normal diurnal variation is often lost in Cushing's syndrome and this may be of diagnostic importance especially when values lie within the normal range. Prunty (1960) reported that in his 12 Cushing's cases, plasma levels of between 3 μg and 56 $\mu\text{g}\%$ were obtained; only in 6 cases was the upper limit of normal exceeded. This worker feels that in the interpretation of plasma values, allowance must be made for the rapidity with which the level may fluctuate from time to time as a result of emotional and other stimuli or from unknown causes. For this reason many feel that techniques involving the measurement of excretion of metabolites over a long period, usually 24 hours, are of greater value in presenting an integrated picture of adrenal activity.

Tests of adrenal stimulation and suppression have been frequently used either to confirm the diagnosis or to differentiate adrenal tumour from hyperplasia. It is believed that patients with Cushing's syndrome due to hyperplasia are extremely responsive to stimulation with ACTH; those with adrenal adenoma show a lesser degree of responsiveness, whilst those with carcinoma are likely to be even more unresponsive because of their autonomous nature /

nature (Lindsay et al 1956; Nabarro et al 1958; Birke et al 1960). Suppression tests have been carried out with cortisone, 9 α -fluorohydrocortisone, dexamethazone, and more recently with SU4885 (Liddle 1959). While the first three suppressors operate by way of inhibiting ACTH secretion, SU4885 operates by inhibiting the synthesis of cortisol at the stage of 11-hydroxylation. This results in a rise in ACTH secretion and a concomitant increase in the urinary 11-deoxy cortisol (Compound S). If the tumour is autonomous, the expected rise of substance S in urine does not occur. Even in these differential tests, exceptions such as patients with adenoma responding to ACTH stimulation and cases with hyperplasia not responding to cortisone suppression have been reported (Brooks et al 1957; Dyrenfurth et al (1960) suggesting that these tests cannot be used with certainty to distinguish between hyperplasia, adenoma and carcinoma; although the type of response observed may reveal adrenal abnormality when compared with the normal response.

On the basis of the various adrenal function tests (17-KS, KGS, Plasma F, ACTH Stimulation, Dexamethazone suppression etc.) carried out on the 13 patients investigated as possible Cushing's cases (Table 15), the production rate by ^{14}C dilution technique provides the best index of adrenal activity. Eight of the 13 cases have been found to have a raised production rate with a range of between 22.6 mg and 112.0 mg/day. A much higher production range has been reported by Prunty (1960). In his 12 Cushing's cases, a production of between 39 and 420 mg/day was encountered - the two highest levels of 210 and 420 mg/day occurring in patients who had bronchial carcinoma with metastases. The great advantage of the ^{14}C test over the 17-KGS, which is its nearest competitor as an index of adrenal function, particularly in Cushing's syndrome is /

is the significantly high production rate encountered in the face of a normal or moderately elevated 17-KGS - values which are convincing enough for the clinician to warrant positive - as against conservative - treatment immediately.

Hirsutism.

The excretion of urinary steroids in hirsutism has been thoroughly investigated more recently by Prunty et al (1958), Brooks et al (1960) and Brooks and Prunty (1960). These workers, in their investigations of the occurrence of hirsutism and virilism in women after the onset of puberty, differentiated between three types of hirsutism: simple hirsutism, adrenogenital syndrome manifest after puberty and Stein-Leventhal syndrome. This differentiation may be made on clinical grounds and on urinary steroid investigations before and after corticotrophin stimulation. According to Prunty (1960), the clinical features which may be suggestive of adrenogenital syndrome are usually shortness of stature with broad shoulders, small mammal and clitoral enlargement; those suggestive of the Stein-Leventhal group are prolonged intervals between menstrual periods or amenorrhoea, anovulation and sterility with some degree of ovarian enlargement demonstrable clinically or radiologically. Other hirsute cases not belonging to either of the two groups just described are referred to as idiopathic hirsutism and very often the features which differentiate this group from the other two groups are mental depression and a family history of hirsutism.

Biochemically, all three groups may show some elevation of urinary 17-ketosteroids (but always below 30mg/day) and no clear distinction can be made on this basis. However, from the figures of Brooks and Prunty (1960), the increase appears to be greatest in the adrenogenital type, less in the simple hirsute group and /

and least in the Stein-Leventhal group. All three groups showed an exaggerated response of 17-ketosteroids to ACTH stimulation - the exaggeration being least in the adrenogenital group. The 17-ketogenic steroid excretion in all three groups was within normal limits and the response of these steroids to ACTH was comparable with the normal response; and again the adrenogenital group showed the least response comparatively. The best test to differentiate between the three groups appeared to be the measurement of the resting urinary pregnanetriol excretion. In this respect, the adrenogenital group showed elevated values of 4-10 mg/day as compared with 1-3 mg/day excreted by the other two groups (normal being 1-2 mg/day). The high pregnanetriol excretion was interpreted as being the result of a deficiency of 21-hydroxylation of 17-hydroxyprogesterone in the synthesis of hydrocortisone. Under ACTH stimulation, the excretion of this steroid was markedly increased in the adrenogenital type as expected, but the response in the other two groups was variable.

From all these results together with those obtained from a detailed study of the individual urinary 17-ketosteroid excretion before and after ACTH stimulation, Brooks and Prunty (1960) suggested that in post pubertal hirsutism, there is one type which is analogous to congenital adrenal hyperplasia in which there is a relative inability to synthesise cortisol with a consequent compensatory increase in corticotrophin production. In this clinical condition, there is a relatively small excretion of cortisol metabolites in the urine with a concomitant increased excretion of pregnanetriol, 17-hydroxy-pregnanenolone, 11-oxopregnanetriol and 11-hydroxyandrosterone. Furthermore, following ACTH stimulation the response in the excretion of cortisol metabolites is poor but that of pregnanetriol excretion is markedly elevated and therefore of diagnostic importance. /

importance. Patients with simple or idiopathic hirsutism have a tendency to excrete relatively more of the 17-ketosteroids derived from C₁₉ precursors compared with those derived from cortisol and results, particularly from the study of pregnanetriol excretion rates before and after ACTH stimulation, seem to indicate that the underlying cause is not due to an impairment of cortisol secretion with a compensatory increase in corticotrophin but to a potentiation of the production of adrenal androgens. In the Stein-Leventhal group, the steroid excretion patterns which are similar to those of the simple hirsute group, suggest that besides ovarian there is also an adrenal involvement since upon stimulation with ACTH, the increased 17-KS found are due mainly to the increase in the dehydro Δ^4 androsterone and 11-hydroxyandrosterone fractions which appear to be exclusively of adrenal origin.

Against a background of such useful information on hirsutism and in the absence of data from ACTH stimulation and pregnanetriol excretion, no attempt was made to categorise the 7 cases investigated into the group each ought to fall. But data on Table 17 show that six out of the seven hirsute cases registered a normal hydrocortisone production rate by the urine method. Furthermore, in the absence of a normal production range - for women of comparable age group - by the blood method, five of the cases registered a subnormal production when evaluated on the basis of a normal range of 16 - 30 mg/day. Whether these blood production values are normal values or indeed subnormal values, the answer seems to lie in the hands of the research worker who may be fortunate enough to obtain, for comparison, a normal production range from healthy adult female volunteers. However, the striking feature in all these cases investigated is that their miscible pools (A) were generally found to be larger than normal;

normal; but because their turnovers (K) were smaller than normal as judged by a biological half life of greater than 2 hours (Normal 60 - 90 mins.), the calculated production rates appeared to be subnormal. The presence of a large miscible pool in these hirsute cases, in the absence of adrenal hyperactivity, seems to suggest a delayed metabolism of the circulating steroid and this would account for the diminished turnover rate of hydrocortisone. But a subnormal estimate of production by the blood method and a normal estimate of production by the urine method are still incompatible. Certainly, further investigations using the blood method should be rewarding in throwing more light on this apparent delayed steroid metabolism and in deciding once and for all the true pattern of hydrocortisone production rates in the various types of hirsutism.

Comparative Indices of Adrenal Function.

The degree of sensitivity of the various adrenal function tests may be judged from the findings on Tables 15, 16, 17. It will be noted that the production rate studies by isotope dilution are the most reliable. In an attempt to evaluate all these tests, Cope and Black (1959) found that there is very poor relationship between the hydrocortisone production rate and the 17-KS excretion rate. They cited instances when the 17-KS output was 25 mg/day, the production rate was 10 mg/day; and that an output of 30 mg/day occurred in a patient when his production rate was 225 mg/day. This poor correlation, though not so dramatic, also occurred in the Cushing's cases reported on Table 15. With the 17-KGS, the relationship was reported to be more encouraging. At high rates of adrenal activity the excretion of 17-KGS is approximately half that of the daily production, but in low normal or moderately raised adrenal activity

activity the relationship is poor. In this research, of the eight Cushing's cases with elevated production rates, 5 registered a raised 17-KGS and 2 a raised 17-KS. When the production rates were normal or diminished as illustrated on Tables 16 and 17, the 17-KS and 17-KGS excretion values were also normal or diminished. But the extent of increased or decreased values was greatest in the production rate method.

Other Production Rate Values.

Since the introduction of this ^{14}C test for adrenal function, the hydrocortisone production rates in many clinical conditions have been investigated. Cope and Black (1958) reported that in a series of 12 tests on persons undergoing adrenal stimulation with AOTH, the mean recovery of the administered ^{14}C was 76.4% in 24 hours and the hydrocortisone production ranged from 22 mg to 257 mg/day with a mean of 99 mg/day. In contrast, a series of 4 cases of gross hypoadrenalism due to hypopituitarism or Addison's disease gave a mean 24 hr excretion of 81% with a daily production of 0.8 to 1 mg. In a series of six active disease cases (lymphosarcoma, carcinoma breast, (2) rheumatic fever, advanced tuberculosis, advanced Hodgkin's disease), the cortisol production ranged from 10 - 25.8 mg/day with a mean of 18.1 mg as compared with a daily mean of 12.8 mg, suggesting that in these active disease states, some adrenal stimulation had occurred. In three other subjects with well marked hyperthyroidism, the mean production of 21.9 mg/day pointed to a greater degree of stimulation and this is not surprising in view of the increased metabolism in these patients. In three patients with advanced hepatic cirrhosis the mean estimate was 6.7 mg/day, a figure suggesting that the output in severe liver disease is reduced to some 50 or 60% of the normal./

SUMMARY of RESULTS.

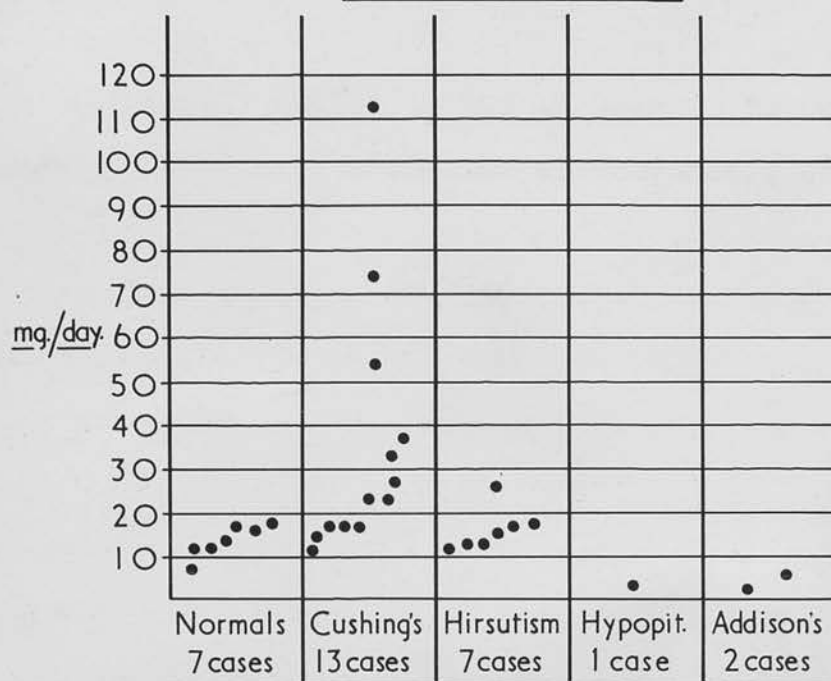


Figure 19: Hydrocortisone production rates from Urine studies.

SUMMARY of RESULTS.

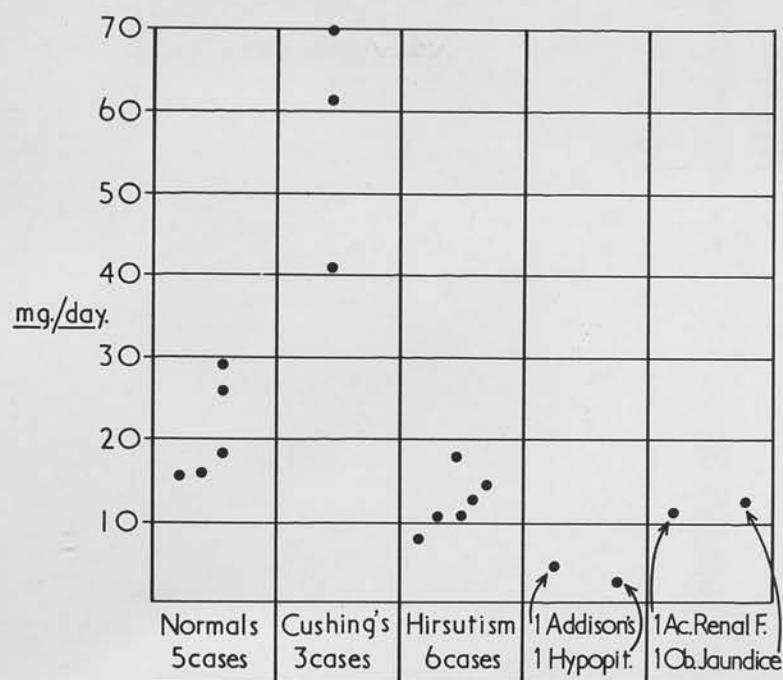


Figure 20: Hydrocortisone production rates from Blood studies

normal. In 1959, these same workers studied the production rates in a series of 8 women during the last month of normal pregnancy and reported a production of between 20 and 40 mg/day indicating that in late pregnancy the production is increased from 2 to $2\frac{1}{2}$ times the non pregnant figure.

Peterson (1959) also recorded similar increases by the method of blood determination during ACTH stimulation and in hyperthyroidism. A reduced production was observed in myxedema, in cirrhosis and in prolonged salicylate or oestrogen therapy. To this interesting pool of production rate values are now added further information obtained from this research regarding the production rates in Cushing's syndrome, in hirsutism, in some hypoadrenal states and in the post surgical state. This last named condition is the subject of investigation and discussion of the next chapter.

To close this chapter, an attempt is made to summarise the results of all the investigations carried out thus far by means of some graphical representations. Thus, figure 19 summarises the production rates measured from urine studies and Figure 20, those from blood studies.

Chapter IV : The adrenal and metabolic response to Surgery

The adrenal gland and the metabolic response to surgery.General.

From previous work undertaken in this laboratory, it has been observed that the usual changes which occur in the immediate period following surgical operation are 1) the occurrence of oliguria, 2) the increased plasma levels of hydrocortisone and excretions of urinary steroids and 3) a sodium retention with a concomitant potassium and nitrogen depletion. It is currently believed that these metabolic changes are necessary and have been designed by the body to maintain osmotic equilibrium between the various fluid compartments, to withstand stress and to provide the energy and raw materials for repair and healing of the wound.

The occurrence of oliguria, suggesting involvement of the kidney and alterations in water and electrolyte metabolism, was known to occur following surgical operations over fifty years ago (Pringle 1905) but little importance was attached to this observation then. Stewart (1955) suggested that this lack of interest might have been, in part, due to the belief that a low urinary volume only indicated a reduced intake of water and in part due to the technical difficulty of studying water and electrolyte metabolism at that time. But later on when it became evident that metabolic changes, other than oliguria, did indeed occur - mainly from the urinary excretion studies of nitrogen, potassium and sodium - much interest was devoted to these phenomena with a view a) to formulate a rule in the management of the post-surgical patient and b) to investigate the mechanism by which these metabolic changes operate. As a result of such research carried out by numerous workers from both sides of the Atlantic, many of the metabolic changes and knowledge concerning the utilisation /

utilisation of fluid, electrolytes, carbohydrates, nitrogen and fat in the immediate post-operative period are now well documented.

But the problem is by no means solved yet; for one, the mechanism responsible for the changes involving electrolyte and nitrogen balance is still uncertain and for another, the alleged increased secretion of adrenal corticosteroids from urinary excretion and plasma level investigations post surgically, still needs to be supported whenever a more reliable test for adrenal function is available. Thus, this section of the thesis is concerned with the measurement of the endogenous hydrocortisone production before and after surgery and, if possible, to interpret the role of the adrenal gland in the light of other metabolic changes simultaneously measured.

The adrenal response to surgery.

One of the earliest observations suggesting that the urine of post-operative patients contained increased amounts of cortin-like activity was made by Weil and Brown (1939, 1940). These workers injected the extracts of such urine into adrenalectomised rats, subjected them to a cold environment and measured their mean survival time. The increased survival time observed was taken to mean the presence of increased activity in the urine extract and therefore manifestation of increased adrenocortical activity as a result of operation.

Evidence that human urine did in fact contain a substance or substances with some of the properties of the hormones of the adrenal cortex came from the works of Perla and Marmosten - Gottesman (1931), Grollman and Frior (1932) and Anderson et al (1938). These workers injected the benzene-soluble material from urine into adrenalectomised rats and observed that the same effects could be brought /

brought about by injection of adrenal extracts. Dorfman (1943), in a series of investigations, showed that the extracts of pooled urine had all the biological activities of extracts of adrenal cortex, namely, that urine extracts could - in the adrenalectomised rats - maintain life, protect against cold, deposit liver glycogen and protect against water intoxication. A year later (1944b), these workers reported that the extracts of urine from seven Addisonians showed no demonstrable activity; that although urine extracts of monkeys (1944a) with intact adrenals or after castration contained substantial adrenocortical activity, this activity was lost when they were adrenalectomised. The information from all these investigations strongly suggested that the active principles in the urine extracts were, in fact, hormones which were mainly elaborated, not by the testes or ovaries, but by the adrenal cortex.

Venning et al (1943,1944), using the life maintenance property of urine extracts of post-operative patients and applying the cold protection and liver glycogen deposition tests as indices of adrenal function confirmed the earlier observations of Weil and Brown. They concluded that post-operative patients excreted as much as thirty times the amount of cortin-like substances as compared with normal persons.

Shipley et al (1946), reviewing the evidence of increased adrenocortical activity after operation, concluded that, despite the poor specificity and recovery given by the biological methods then in use, the large amounts of cortin-like substances constantly found in urine after surgery was convincing enough to suggest increased adrenocortical activity.

The decrease in the number of circulating eosinophils often observed by many workers in the immediate post-operative phase /

phase was also considered to be suggestive of increased activation of the adrenals. Roche et al (1950) observed that the number of eosinophils fell rapidly within one or two days following operation and that in a few cases, they disappeared completely. The severity of the operation seemed to have a bearing on the intensity of response. The conclusion that this response was solely due to increased adrenocortical activity becomes doubtful when it is now realised that increased secretion of adrenaline at the time of surgery can also cause an occurrence of eosinopaenia.

The excretion pattern of urinary 17-ketosteroids following operation has been observed widely. Forbes et al (1947) reported that the increase, when it did occur, was evident 24 or 48 hours after operation. Subsequently a gradual fall in output occurred and four or five days postoperatively the values were frequently below the preoperative range. The return to normal values was variable and occurred some 7 - 10 days after operation. Similar reports were forthcoming from the investigations of Perry and Gemmell (1949), Venning and Browne (1947), Mason and Engstrom (1950), Hardy and Rawdin (1952) and Birke et al (1955).

With the introduction of improved methods for the estimation of adrenal steroids in biological fluids, further evidence became available. In 1953, Thorn et al, using a method which measured both the free and conjugated 17-hydroxycorticosteroids, reported finding increased values in urine of patients after major operation. These workers also added that minor operations evoked no such increase. Tompsett and Smith (1954) investigated the urinary excretion patterns of 17-KS, 17-KGS and ASFS (acid stable formaldehydogenic steroids) before and after surgery. Their results from measurement of 17-KS and 17-KGS indicated a variable response, but those based on the /

the measurement of ASFS showed a definite 2 to 3-fold increase after operation.

An increase in the excretion values of tetrahydrocortisol (THF) and tetrahydrocortisone (THE), the two major urinary metabolites of hydrocortisone, was reported to occur after major surgery by Cope and Hurlock (1954). The measurement of these two β -glucuronidase hydrolysable steroids as an index of adrenal function was also used by de Courcy et al (1953) who, from 10 normal male urines, found normal mean values of 212 ug/24 hr for THF and 1.5 mg/24 hr for THE. Gold et al (1958) studied the excretion ratios of these metabolites in surgery (and in a variety of other conditions) and reported finding increased values of THF and THE.

In a somewhat extensive study of the adrenocortical response to surgery, Reece et al (1957), observed increased excretions of 17-OHCS in the postoperative period lasting from 2 - 7 days. Of their 48 patients undergoing major and minor surgery, 19 were submitted to partial gastrectomy, 16 to inguinal herniorrhaphy and 13 to laparotomy. The total urinary 17-OHCS were estimated by the Norymberski technique and a pre-operative upper limit of 16.2 mg/day was used for comparison with the post-operative excretion values. Following partial gastrectomy there was an increase of corticosteroid output with a mean peak excretion of 40 mg/day occurring usually on the third post-operative day. The response in inguinal herniorrhaphy was less marked with a peak mean of 25 mg/day and that following laparotomy was very variable.

Likewise Markley et al (1960) measured the urinary corticosteroid output in 29 patients who underwent major surgery. 17 OHCS were measured by application of the Porter Silber reaction and 17-KS by the microtechnique of Vestergaard (1951). Over 75% of /

of the cases showed elevated excretions of 17 OHCS after operation but the excretions of 17-KS on the other hand were rarely elevated above the preoperative value. The administration of AOTH in the post-operative period produced further increases in the excretion of the urinary corticosteroids and this finding strongly suggested that adrenocortical response was not maximal even after major surgery.

When Nelson and Samuels (1952) described their satisfactory method for the estimation of plasma 17 OHCS much useful information was obtained from measurement of plasma steroids before, during and after operation. Franksson et al (1954) measured the plasma levels of 27 patients undergoing surgical operation and reported that on the morning of the day of operation, the plasma steroid levels were usually slightly elevated and that following the preoperative preparation, the level further increased. They attributed this immediate preoperative increase to the stimulation of the adrenal glands caused by the additive effect of tension, preoperative treatment and anaesthesia. Immediately following surgery, all their patients showed a marked elevation of blood 17-OHCS. A peak level was observed to occur some time between 24 and 48 hours after the operation. This was followed by a rapid fall to subnormal values, but returning to normal within 72 hours following operation. Similar information was forthcoming from the laboratories of other workers (Sandberg et al 1954; Elman et al 1955; Mittleman and Barker 1956; Sandberg et al 1956; Helmreich et al 1957; Marks et al 1959; Markley et al 1960).

More direct evidence of increased adrenal activity post-operatively comes from the work of Hume and Nelson (1954). These workers observed an actual increase in adrenal venous output of 17 OHCS after operation in dogs; control values being obtained by leaving the cannulae in situ and measuring the blood levels of steroids /

steroids during the convalescent period. Hardy et al (1957), by means of timed collections of adrenal vein blood also found higher levels of hydrocortisone secretion in patients at operation.

The importance of the liver in the removal of hydrocortisone from the circulation has already been discussed in the previous chapter. Other facts which need to be added in this respect may include the finding of Nelson and Harding (1952), who having injected cortisone intravenously into dogs, found that the steroid was removed very rapidly from the pool and pre-injected levels of 17 OHCS were observed sixty minutes later. These workers also found a marked difference across the liver when arterial and venous blood samples were assayed for steroids. Tomiza et al (1954) found that after an injection of cortisone into intact and hepatectomised mice, blood steroid levels rose in both groups, but the rate of disappearance of the injected steroid from the circulation was slower in the hepatectomised group. With the finding that blood contained β -glucuronidase hydrolysable steroids by Bongiovanni et al (1954) and evidence of inactivation of physiologically active adrenal steroids when incubated with sliced rat or human liver (Reaven 1955), the role of the liver in the metabolism of adrenocortical steroids became established. To test this role further, Tyler et al (1954) measured simultaneously the liver function by bromosulphthalein excretion and the increases in free blood steroids in their patients undergoing operation. Their results suggest that a relationship indeed exists between the condition of the liver and the level of free blood steroids; the greater the liver damage, the greater the steroid rise after surgery.

The administration of anaesthesia during the preoperative preparation being responsible for a slight increase in blood steroid levels was mentioned earlier in this chapter. In a detailed /

detailed study of the effects of different anesthetics on blood 17 OHCS, Virtue et al (1957) showed that 15 out of their 35 patients who received ether as the anesthetic registered a definite increase, but of those who received cyclopropane or thio-penthol-nitrous oxide or spinal anesthetic, only a small proportion showed similar increases; thus pointing out that anesthetic agents differ in their effects, presumably, depending upon the effect the agent exerts upon liver function.

The conjugated blood steroids were also reported to increase following surgery. Hardy and Turner (1956) reported finding a relatively greater increase in the conjugates than in the free steroids post-operatively, implying an increased rate of liver conjugation. Helmreich et al (1957), on the other hand, in a study of 12 patients undergoing major surgery, observed a variable response in the conjugate levels. In some patients a pronounced rise was noted at a time when the free 17 OHCS reached peak levels and in others, there was a virtual absence of such response. This finding suggested that liver conjugation of the steroid was impaired in some patients following surgery but not in others. More recently Steenburg et al (1961) compared the response of serum free and conjugated 17 OHCS in normal subjects under ACTH stimulation to the response in patients after subtotal gastrectomy. They observed a) that the rise in the serum level of the free steroid was more rapid following gastrectomy than that following adrenal stimulation in the normal subject and b) that the rise in the serum level of the conjugates was more gradual in the surgical patients. From these interesting observations, these workers suggested that the elevated blood levels of free steroids following surgery are due in part to delayed metabolism and in part to stimulation of the adrenal cortex.

An interesting but totally different kind of response was observed to occur during hypothermic operations. Swan et al (1957) measured the free and conjugated 17 OHCS in plasma during the course of anesthesia, body cooling, hypothermic surgery and post-operative recovery in a group of 21 patients undergoing major cardiovascular surgery. The pattern of adrenocortical response obtained, suggested that the induction of hypothermia by ice water immersion produced a moderate activation of the adrenal cortex, but when hypothermia was established, even severe trauma failed to produce additional increase in adrenocortical secretion; a finding which is in direct contrast to the changes observed during surgery in normothermic patients. Upon rewarming however, the levels of the free steroids promptly rose but those of the conjugates remained unchanged. These authors suggest that the failure of the plasma 17 OHCS to rise during surgery resulted from a suppression of adrenocortical secretion due to hypothermia. This concept, they added, was supported by the fact that hypothermic dogs also demonstrated a marked decline in adrenal blood flow, in the rate of cortisol output from the adrenal gland and its ability to respond to ACTH stimulation.

The evidence accumulated during the last decade concerning the adrenocortical response to surgery strongly suggests 1) that major surgical operations augment the activity of the adrenals by way of an increase in endogenous ACTH secretion; 2) that adrenal activity is not maximal under these conditions; 3) that diminished liver metabolism following surgery exaggerates plasma steroid levels and brings about a somewhat delayed excretion of steroid metabolites in urine; and 4) that during the hypothermic phase, adrenal activity is suppressed due to its inability to respond to ACTH stimulation.

Nitrogen, water and electrolyte metabolism following Surgery.

Nitrogen.

Following the occurrence of oliguria, the next metabolic change which attracted attention was the constant finding of increased nitrogen excretion in the urine of the post-operative patient. To Cuthbertson must go the credit of being the first to study systematically the metabolism of nitrogen following trauma. In 1930, he observed that increased amounts of nitrogen were excreted by his post-operative and post-fracture patients. During this period of increased nitrogen loss which persisted from 7 - 10 days, he also studied the excretions of phosphorus and sulphur from which he concluded that the source of these increases was the tissue protein. This period of excess protein oxidation was referred to as the "protein catabolic phase." The effects of immobilisation and starvation as possible causes for this increased nitrogen loss were also examined and it was found that they contributed very little; a finding later substantiated by Howard et al (1944). In 1932, Cuthbertson reported that anaesthesia alone did not produce a marked nitrogen loss. Furthermore, although there appeared to be some correlation between the extent of injury and loss of nitrogen, he believed that this increased loss was not solely due to tissue breakdown in the traumatised area, but the result of a generalised rapid protein catabolism to provide material for tissue repair.

Another concept to account for this post-operative nitrogen loss was introduced by Albright (1943). This worker postulated that the increased nitrogen excretion after stress of any kind was due to an imbalance of hormones; namely, that during this period, the anti-anabolic 'S' hormone was greatly in excess of the anabolic /

anabolic "N" hormone and that ingested protein was not utilised, the amino acids passing through the liver and being excreted by the kidneys. To this view, Peters (1944) and Grossman et al (1945) also subscribed. But the finding by Wilkinson et al (1950) that urea and not amino acid was the major nitrogenous constituent in the urine in the post-operative period rendered this anti-anabolic concept questionable if not invalid.

Werner (1948) on the other hand maintained that the increased nitrogen excretion was due to starvation since in the immediate post-operative period the nutritional intake was either absent or invariably inadequate. And although a few years earlier (1944) Co Tui et al claimed to have abolished the negative nitrogen phase after gastrectomy by the administration of large quantities of amino acids and protein hydrolysates, Wilkinson et al (1950) were unable to confirm Co Tui's findings. These last mentioned workers support the view that the increased nitrogen loss following operation was due mainly to the effect of trauma on the metabolic processes and that the food restriction in the immediate post-operative period contributed very little to it.

It is now believed that the extent of nitrogen loss after operation depends upon the nutritional state of the patient. Many workers (Levenson et al 1945; Wilkinson et al 1950; Moore and Ball 1952) have reported that patients who have lost weight pre-operatively as a result of malnutrition or disease, excreted less nitrogen post-operatively than those who were well nourished. It would thus appear that to starve the patient for some time before the operation is the most effective way of abolishing or minimising the post-operative nitrogen loss. Indeed, earlier attempts to abolish completely the /

the catabolic phase succeeded only in increasing the gross amount of nitrogen excreted but did not alter the overall negative nitrogen balance. Stewart 1955, among other workers, is inclined to believe that this "catabolic phase" following surgery cannot be abolished; that it represents an inherent response to injury and is a physiological rather than a pathological process which should not be discouraged or interfered with.

Oliguria.

The early belief that a decreased urinary volume following surgery, only reflected a reduced intake along with a large fluid loss at operation led to the practice of saline administration routinely to all surgical patients. When later it became evident that these patients were not able to tolerate large volumes of infused saline and often developed oedema, sometimes with fatal results, Coller et al (1944), who earlier on proposed their 'clinical rule' as a rough guide regarding the amount of saline to be administered to the surgical patient, strongly criticised the evil effects of excess saline administration and advocated the discontinuation of the practice of the old rule.

Many workers have observed that the severity of the operation bore some bearing on the extent and duration of the oliguria; the more severe the operation, the more marked and prolonged the period of oliguria. Even when blood transfusions, calculated to replace the blood lost, were given at the time of surgery, oliguria was seen to occur. Cooper et al (1949) reported that specific gravities of the post-operative urines were of the same order as those in which no attempt had been made to replace the fluid lost. This clearly showed that the oliguria did not appear to be entirely due to the /

the state of hydration of the patient.

That the effects of anesthesia were not the cause of the oliguria was shown by Collier et al (1943) and by Mayer (1950). From an extensive study of the mechanisms of the kidney in an anesthetised person not subjected to operation, these workers were able to demonstrate that there was no deleterious action of the anesthetic upon the kidney parenchyma.

A decrease in the glomerular filtration rate and renal blood flow to account for the oliguria was suggested by Elman et al (1949) who obtained a diminished creatinine output following operation in their patients. Habib et al (1951) further suggested that consequent upon this diminished filtration rate, there was also the tendency of increased reabsorption of water by the renal tubules to occur. Ariel and Miller (1950), while agreeing that the oliguria was not due to any intrinsic renal dysfunction or renal damage, suggested that the primary cause of the decreased glomerular filtration and renal blood flow was a result of hormonal imbalance following surgery.

Sodium.

The occurrence of a low or even absent urinary sodium in patients after operation had been reported by Robinneau (1933) and like the oliguria which occurred at the same time, this fall in sodium output was thought to be due to the conservation of the ion by the kidney as a result of diminished intake. However, many workers among whom Moore and Ball (1952) and Renwick et al (1955), observed that if common salt were withdrawn from the diet, it took several days before a low urinary sodium could be effected. Earlier on, Wilkinson et al (1949), observed that the period of sodium retention, occurring within 24 hours after operation and /

and lasting for several days, roughly coincided with the catabolic phase and that this retention persisted even when quantities of sodium chloride, comparable to those given during the pre-operative period, were administered orally or intravenously. All these findings suggest that the post-operative sodium retention cannot be explained on the basis of a reduced intake.

It must have been tempting to suggest that the post-operative oliguria which consequently lead to a dilution of the extracellular fluid was in fact responsible for the diminished sodium output. That this was not the case or that the retention of sodium and water were two distinct phenomena became manifest when Limbert et al (1945) and Cooper et al (1949) reported that sodium excretion was greater in the oliguric phase than in the succeeding phase when a very dilute urine was voided. This distinction was confirmed when Holland and Stead (1951), undertaking to investigate the effects of pitressin injections on the excretions of water, sodium, potassium and chloride, reported that while there resulted a marked oliguria due to increased tubular reabsorption, there was no demonstrable effect on the excretion of sodium and other electrolytes.

The retention of a great quantity of sodium in the days immediately following operation will cause a disturbance in osmotic balance which in turn will result in a major rearrangement of the body fluid and electrolyte distribution. In the light of a potassium loss - which will be discussed later - occurring during the same period, it is conceivable that a shift of fluid and ions will take place between the intracellular and extracellular spaces, starting as a localised reaction in the injured region and spreading to involve other areas. Winfield et al (1951) analysing the sodium and potassium content in the injured muscle in post-operative /

post-operative patients observed that the sodium content was greater and potassium content less than expected from the amount of oedema fluid present. These workers suggested that as a result of altered cell membrane permeability following injury, the retained sodium passed into the cells liberating potassium. This concept of ionic exchange gained confirmation from the work of MacPhee (1953) who analysed the electrolyte content of red blood cells in patients before and after major operation.

When it is considered that the period of increased potassium output ceases at the end of 72 hours after operation and that sodium retention continues for several days more, then this exchange of sodium for intracellular potassium appears to play only a minor role in the sodium retention following operation. Le Quesne and Lewis (1953), cognisant of this dissociation in time between the potassium and sodium response to surgery, distinguished an early retention of sodium and water in the first 24 hours after operation from a late sodium retention which might coalesce with the period of early retention or might be separated from it by a 24 hour period of negative sodium balance. These workers pointed out that the late sodium retention may be enhanced by potassium deficiency in view of the work of Black and Milne (1952). From a detailed study of their 21 patients undergoing surgery, Le Quesne and Lewis concluded that the primary water retention was due to the increased secretion of antidiuretic hormone and that the periods of sodium retention were partly or wholly due to the increased secretion of adrenocortical hormones in response to surgical stress.

Potassium.

Like nitrogen, an increased urinary potassium following operation was noted by Outhbertson (1930) and this occurrence was thought to be a necessary accompaniment of the post-operative nitrogen excretion in view of the work of Benedict (1915) who observed that in starvation, the nitrogen loss accompanied by potassium loss was in a ratio corresponding to that of tissue protein which is 10:1.

During the years that followed, many other workers (Howard 1946; Blixenkrone-Moller 1949; Wilkinson et al 1950) also reported increased output of urinary potassium following surgery. But the interesting points brought to light were that this output occurred much earlier than the increased breakdown of the catabolic phase and was maximal within the first 24 hours; that the excretion was much greater than expected from the nitrogen output and that it occurred even when K intake was restricted during the 3 days following operation. Stewart (1955) further reported that the quantity lost was variable but could amount to several grams and bore no relation to the nitrogen output; that the loss began six hours after injury and that its duration was much shorter than the period of sodium retention. From all these data, he suggested that this early post-operative potassium loss was neither due to a direct consequence of the catabolic destruction of cellular protein nor a response of sodium retention but a very early part of general reaction to trauma.

Working with mice Rosenthal and Tabor (1945) reported that the potassium content in the injured hind leg was lower than that in the uninjured counterpart and that this decrease accounted for only part of the total amount excreted, suggesting that the difference must have come from undamaged areas. At the same time, a gain in sodium, /

adrenal extracts to adrenalectomised and hypophysectomised rats resulted in liver glycogen deposition and increased nitrogen excretion due to gluconeogenesis of tissue protein.

The role of the adrenal gland as the causative factor in the metabolic response to surgery seemed, at this stage, ripe for formal proclamation. In fact it was Albright, in 1943, who first proposed the causative role of the adrenal hormones to account for the post-operative metabolic changes. This causative concept met with further wide acceptance when it was subsequently observed that such responses did not occur in the absence of the adrenal glands and that an overdose of ACTH or cortisone acetate administration evoked metabolic changes comparable to those following surgery.

Not long later, Ingle (1947, 1951) investigating further the role of the adrenal gland in the production of a negative nitrogen balance and sodium retention after injury, reported that in 2 groups of adrenalectomised rats, one maintained on adrenal hormones and the other on physiological saline, the former group displayed the usual metabolic changes after injury but the latter group did not. These interesting observations suggested that although the presence of adrenal hormones was necessary for the changes under consideration to take place, the increased amounts of adrenal hormones were not themselves responsible for these changes. Ingle therefore suggested that the role of the adrenal gland was a "permissive" one - as opposed to causative - which aimed at maintaining homeostasis rather than initiating the series of events found to occur following surgery.

To test whether the "permissive role" of the adrenal gland also operates in man, work from this laboratory (Stewart et al 1957), Robson et al 1955, 1956, 1959; Dudley et al 1957) has shown that the usual metabolic responses occurred following the removal of /

of the adrenal gland in patients who, since removal of the first had been maintained on a constant dose of cortisone. There was the usual sodium retention, together with increased loss of potassium and nitrogen. But the increase in 17-hydroxysteroids or formaldehydogenic steroids in urine which normally follows operations that do not involve the adrenal cortex did not occur. The possibility that there might be a rapid increase in aldosterone secretion during the second-stage adrenalectomy just before the removal of the adrenal gland and therefore responsible for the metabolic changes was suggested by Llauro and Woodruff (1957). This was checked and the results showed that although there was a considerable rise in urinary aldosterone in the first 48 hours following the first-stage adrenalectomy, this mineralcorticoid could hardly be detected in the urine during a similar period after the second-stage adrenalectomy. The possibility that the metabolic changes might be initiated or maintained by changes in the blood concentration of adrenal steroids was also checked. In three subjects undergoing the second-stage adrenalectomy but maintained on a constant intravenous infusion of 150 mg hydrocortisone throughout the 24 hours, none of them in the first nine hours after operation showed the 3 - to 4 - fold rise in free steroid that occurs almost invariably after operations which do not involve the adrenal cortex. The conclusions to be drawn from all these investigations lend support to the belief that in human subjects also the complex metabolic changes which follow surgical trauma are not due primarily to the increased secretion of adrenocortical steroids including aldosterone and that as Ingle had suggested, the role of the adrenal gland is a "permissive" one since only the presence of these steroids in concentrations above a certain minimum appears to be necessary in order that those reactions /

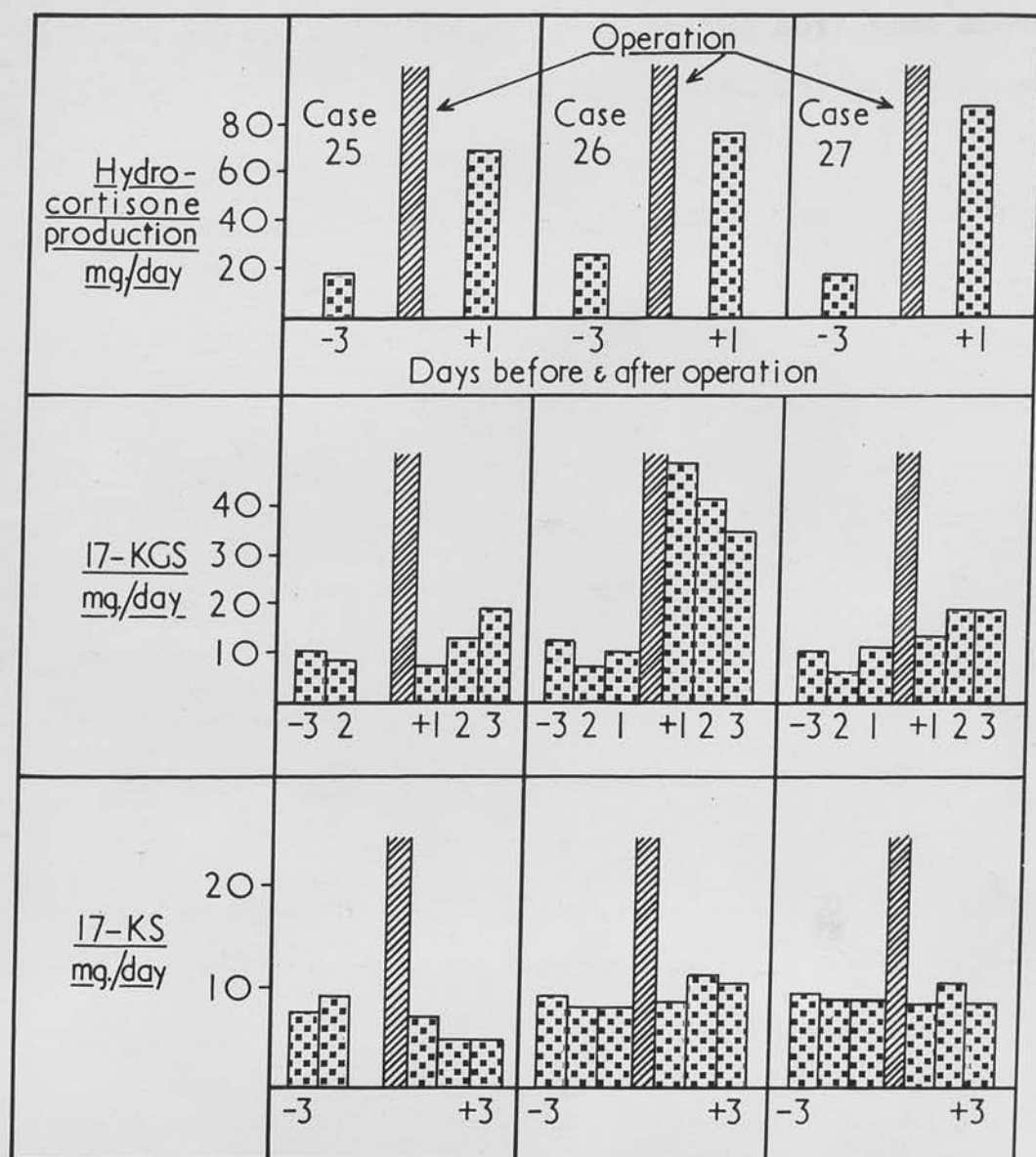
reactions which constitute the metabolic response to injury may occur.

Purpose and Plan of Investigation.

Stewart (1961) has pointed out that although the evidence obtained thus far from observations on adrenalectomised patients suggests that the initiating causes of the response must be sought elsewhere, nevertheless the concept of the "permissive action" of the adrenal gland in this context becomes more convincing if it can be proven 1) that the increased output of corticosteroids after surgery in "normal" patients represents a real increase in hormone production and 2) that there is neither delayed excretion or reduced metabolic destruction of circulating hormone as a result of trauma.

It was with such a purpose in mind that this investigation was undertaken. Production rates were measured before and after operation to ascertain the occurrence and extent of the real increase - if any. At the same time, from the negative slopes of the diminishing specific activities (incident in the measurement of production) together with the measurements of the urinary excretion pattern of the administered radioactive material, it might be possible to deduce whether in fact there was a reduced metabolic destruction or delayed excretion as a result of operation. A total of 5 cases were investigated; four undergoing major operations not involving the removal of the adrenal glands and one undergoing right adrenalectomy after having previously undergone left adrenalectomy. Measurements of 17-ketosteroids and 17 Ketogenic steroids were also carried out every day during the period of study; so were those of urinary potassium, sodium and total nitrogen to reflect the metabolic changes. In most cases the period of study was three days before and three days after /

Figure 21: Adrenal function before and after major operation.



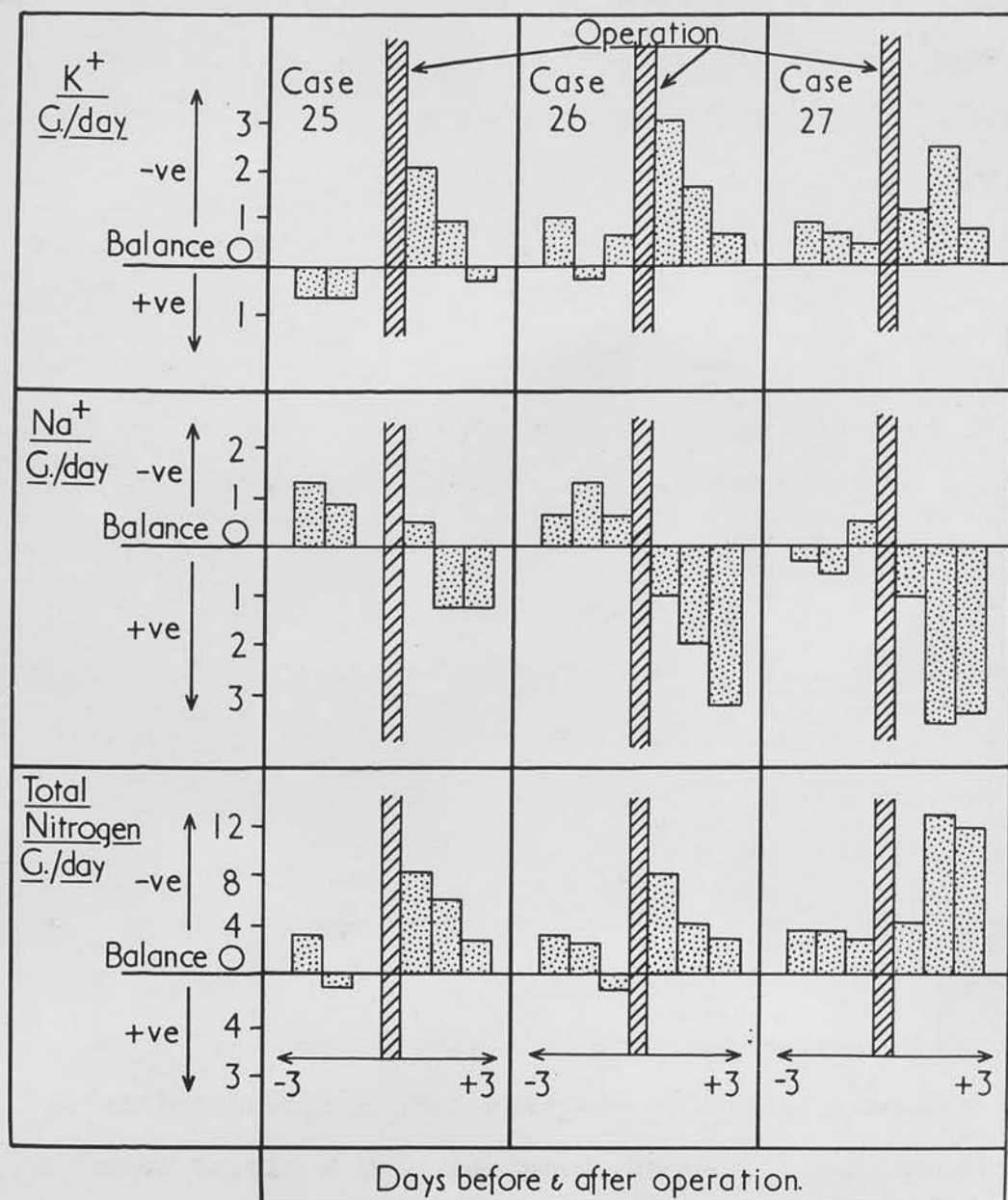
after operation; only in one case was this period extended to 7 days after operation.

To carry out the balance studies of potassium, sodium and total nitrogen, the patients were put on a constant diet beginning a week before the operation and extending beyond the period of study. Their food came from the dietetic kitchen and the diet contained a caloric range of 1000-2000, 0.5 gm NaCl, 1-2 gm K and 55-60 gm protein. This diet was further supplemented with 4 gm of NaCl taken by mouth as cachets so that a salt intake of 4.5 gm daily was maintained. On the day of operation when no food was given 500 ml of physiological saline was administered and this mode of salt administration was continued on the days following the operation if the patient was unable to ingest solid food. However, in four of the five cases investigated, the patients were well enough to begin taking food by mouth on the day after the operation. No attempt was made to collect faeces passed during the period of study simply because there did not appear to be a great variation in the amounts of the relevant substances excreted by this route. But 24 hour urine was collected every day without preservative and stored in a deep freeze until ready to be analysed. Stomach aspirates and bile were also analysed whenever these were collected in the immediate period following operation.

Results.

The results of the first three investigations carried out are summarised on figures 21 and 22. Case 25, an elderly male patient aged 83 underwent gastrectomy due to ? carcinoma. Hydrocortisone production in his case was measured by the urine method and his urinary excretion rates of the administered ^{14}C were 51% and 60% before and /

Figure 22: Balance studies of K^+ , Na^+ & N before & after major operation.



and after operation respectively. His pre-operative production was found to be 15.3 mg/day and that measured one day after operation registered a real increase to 66.6 mg/day. Case 26, a male patient aged 66 underwent gastroenterostomy and vagotomy due to duodenal ulcer. Measurement of hydrocortisone production in this case was carried out by the blood method. A pre-operative value of 26.6 mg/day was obtained from a somewhat large miscible pool of 3.1 mg and a turnover with a biological half time of 114 minutes. The post-operative production measured one day after operation, registered an increase to 73.2 mg/day as obtained from a miscible pool of 4.3 mg and a biological half time of 59 minutes. Urinary ^{14}C excretion rates were found to be 82% and 83% of the administered dose pre- and post-operatively. Case 27, a male patient aged 52 underwent cholecystectomy due to obstructive jaundice. His pre-operative production as measured by the blood method was 13.2 mg/day; this figure was calculated from a miscible pool of 1.66 mg and a biological half life of 122 minutes. His post-operative production showed an increase to 85.2 mg/day as calculated from a markedly increased miscible pool of 10.2 mg and a slope with a biological half life of 120 minutes.

In all the three cases, the production values one day after operation ranged from 67 - 86 mg/day, showing that a real increase in adrenal hormone production had taken place, Figure 21. However, only in one case (case 26) was this real increase reflected strongly by the 17-ketogenic steroid patterns; but a closer examination will reveal that 17-KGS increases did actually occur in the other two cases (case 25, case 27), though not as pronounced from values obtained on the 2nd and 3rd post-operative days. This real increase in production could not be said to have occurred from the excretion patterns of the 17-ketosteroids. In fact, the excretion registered /

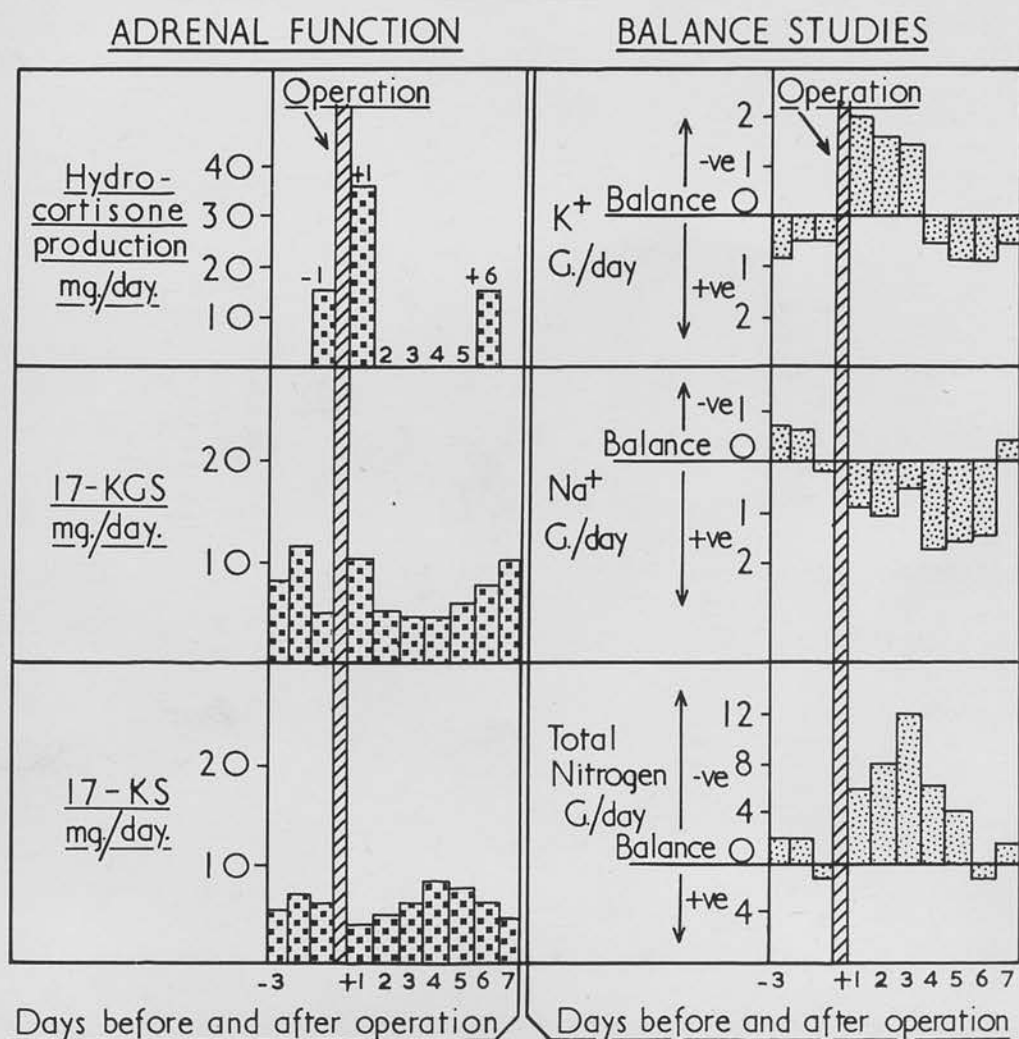
registered by case 25 suggested a decrease in adrenal function, that of case 26 suggested a moderate increase and that of case 27, no change at all in adrenal function after operation.

In all three cases, as judged from the balance studies (Figure 22), it may be said that there occurred a marked potassium and nitrogen loss and an equally marked retention of sodium as a result of operation. The potassium loss began very promptly reaching a peak during the first 24 hours but may be considered to have reverted to normal balance by the third post-operative day. Sodium retention began very moderately but appeared to be marked on the 2nd or 3rd post-operative day. Total nitrogen loss, like potassium, began very promptly after operation, reached a maximum on the first or second post-operative day and in the case of case 27, this loss appeared to continue beyond the 3rd post-operative day.

It seemed worthwhile to investigate how long the increase in hormone production persisted as a result of operation and likewise how long the nitrogen and electrolyte changes continued with particular attention to the sodium retention. Accordingly, for this particular investigation, the period of study was extended to include another 4 days beyond the usual 3 post-operative days; so that a period of study of 10 days was launched, 3 days before operation and 7 days after operation. The patient, Case 28, selected for this investigation was a male aged 60, who underwent gastrectomy due to duodenal ulcer. His pre-operative production was 18.0 mg/day as calculated from a pool of 0.87 mg and a biological half life of 54 minutes, Figure 23. One day after operation, he registered a production increase to 38.4 mg/day as calculated from an increased pool of 2.2 mg and a biological half time of 60 minutes. The increase in production in this case though real, was not as marked as those /

Figure 23: Metabolic response over a longer period following surgery.

Case 28.



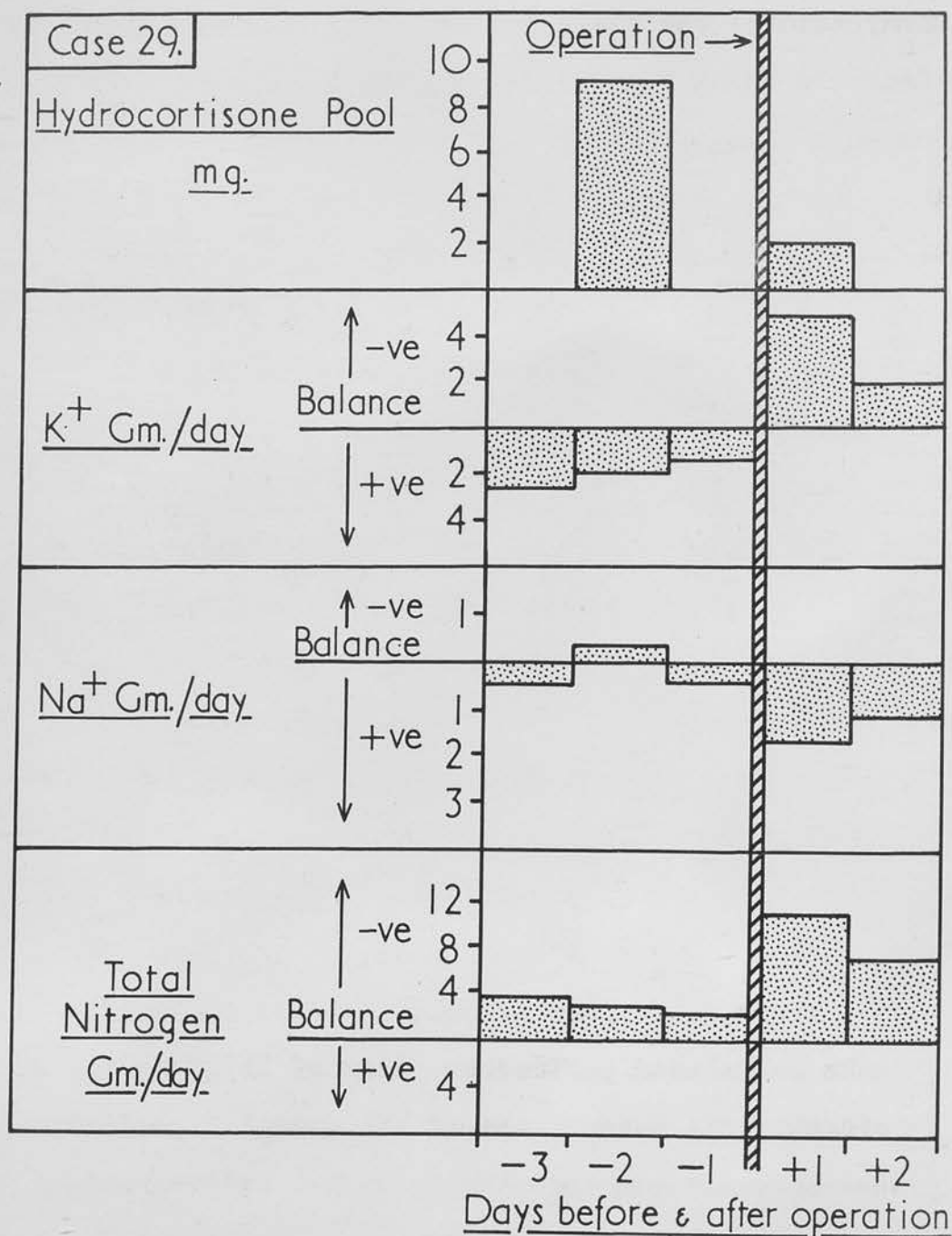
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those in the 3 cases previously cited. This was not surprising since - as the physician and surgeon in charge of the case had suggested - the operation turned out to be less serious than was expected. Another measurement performed on the 6th post-operative day revealed a return to normal production of 16.1 mg/day from a pool of 0.96 mg and a biological half life of 60 minutes. This moderate but real increase in adrenal activity immediately following operation was not reflected at all in the 17-ketogenic and 17-keto-steroid excretion patterns.

Even in the face of this moderate increase in adrenal activity there occurred the usual potassium and nitrogen loss and a concomitant sodium retention due to operation, Figure 23. The potassium loss which was maximal during the 24 hour period immediately following operation reverted to a pre-operative balance after the 3rd post-operative day. The sodium retention, although appeared to have started moderately at first with a tendency to revert to a pre-operative balance on the 3rd day, was most marked on the 4th, 5th and 6th post-operative days and abruptly return^{ed} to a pre-operative balance on the 7th day. The nitrogen loss which started immediately after operation was most marked on the 3rd post-operative day but returned to a pre-operative balance two days later.

Having now established the occurrence of a real increase in adrenal hormone production in patients undergoing operations not involving the removal of the adrenal glands, the next obvious step was to prove the absence of such a production in subjects undergoing operations involving the complete removal of the adrenals. The subject Case 29 selected for this investigation had earlier on been confirmed to be a Cushing's case by the ¹⁴C test and had subsequently had a left adrenalectomy performed on him. The removed gland /

Figure 24: Response after 2nd.-stage ADRENALECTOMY.



gland weighed some 17 Gm and showed evidence of a large diffuse hyperplasia without evidence of mitosis. When he had recovered from the first stage adrenalectomy, he was receiving 25 mg cortisone acetate daily until 5 days before he was due for the second-stage adrenalectomy when he was given 200 mg cortisone acetate daily. 2 days before the operation the blood ^{14}C test was carried out with a view to measure the actual amount of hydrocortisone the remaining adrenal gland was producing and to repeat the test one day after second-stage adrenalectomy to see if there was an absence of such production. The patient was put on a diet regime just as the previously mentioned patients had been so that studies of the metabolic response could also be carried out.

The results obtained from the ^{14}C tests - though shocking at first because it was totally unexpected - revealed some useful information. A pre-operative production of 163.2 mg/day was registered when calculated from a miscible pool of 9.8 mg and a slope with a biological half time of 60 minutes. The one day post-operative test registered a production of 36.0 mg/day as calculated from a reduced miscible pool of 1.93 mg and a biological half time of 59 minutes. It is true the tests revealed a real decrease in apparent production after the removal of the remaining gland as judged from the sizes of the miscible pools (Fig.24); but what appeared to be absurd were the calculated production rates of 163.2 mg/day and 36.0 mg/day. Evidently the measurement of the actual F production of the remaining adrenal gland pre-operatively or the measurement of an "expected" zero production after adrenalectomy could not be achieved even by this isotope dilution method. This is because, during the period of study, the 200 mg cortisone acetate which the patient was receiving every day underwent molecular interconversion such as :

such as : cortisone \rightleftharpoons hydrocortisone.

Therefore, so long as there was an exogenous supply of hydrocortisone in amounts larger than tracer quantities, the measurement of endogenous hydrocortisone production became impossible. Had this inter-conversion not taken place the purpose of this particular investigation would have been completely fulfilled. As it is, because the patient was receiving a constant dose of adrenal hormones throughout the period of study, save only on the operation day when he was given 250 mg instead of the usual 200 mg, the only data which could be used for comparison were the pre-operative and post-operative values of the miscible pool. There was a real decrease of 9.8 mg to 1.9 mg as a result of the 2nd stage adrenalectomy. This decrease could not be attributed to a greater body utilisation post-operatively because the plasma specific activity slopes or the biological half times of the two ^{14}C tests were very similar. It may be assumed then, that the decrease in miscible pool was due solely to the removal of the overactive remaining gland.

In the face of this greatly diminished or nil adrenal activity after operation, there still occurred the usual post-operative metabolic response. The potassium and nitrogen losses together with the sodium retention on the first and second post-operative days were marked enough to lend support once again to the view that the role of the adrenal gland in the metabolic response to surgery is a "permissive" one as had originally been suggested by Ingle and subsequently found to operate in humans from previous work undertaken in this laboratory.

Discussion.

Real increase in hormone production.

This investigation has shown conclusively that a real /

real increase in adrenal hormone production exists as a result of operation and that although a production with a maximum of 85 mg/day was observed to have occurred 24 hours after the operation, it is reasonable to assume that the production between 0 and 24 hours after the operation is greater. This assumption is based on the fact that the highest plasma steroid levels are usually encountered some 12 hours after the operation and these high values will effectively increase the size of the miscible pool which in turn, together with the nature of the slope of diminishing plasma specific activity, will register a higher rate of hormone production. There has been some evidence to suggest that the magnitude of the operation bears a direct relationship with the extent of adrenal hormone production. A post-operative production of 36 mg/day by itself indicates only a 20% increase over the upper normal limit of 30 mg/day but when referred to a pre-operative production of 18 mg/day, the increase becomes significant and represents a 100% increase. The occurrence of this real increase after operation (though moderate when compared with values of 66-85 mg/day encountered in "more serious" operations) was not accompanied by either elevated plasma steroid levels or elevated excretions of 17-ketogenic and 17-ketosteroids. So that, in the light of this finding, earlier reports suggesting that no apparent increased adrenal activity was found to occur after minor operations, simply because there occurred no apparent change in the plasma or urine steroid values must now be accepted with reservations.

Metabolic destruction and Excretion.

That there is a reduced metabolic destruction of the circulating hormone in the immediate post-operative period giving rise to raised plasma steroid levels was discussed earlier in the introductory part of this chapter. This may be true during the /

the period 0 - 24 hours after operation but from the results of the 5 cases investigated here there was no evidence to suggest that during the period 24 - 48 hours after operation there existed either a reduced metabolic destruction of the circulating hormones or a reduced steroid excretion. The slopes of the lines of fit containing the exponentially diminishing plasma steroid specific activities or more practically, the biological half times measured before and after operation were very similar in 4 out of the 5 cases. The excretion rates of the administered dose before and after operation were also of the same order and certainly normal as evidenced by an excretion rate of over 80%.

Production in Adrenalectomy.

It is true that the attempt to measure the activity of the remaining adrenal gland in a patient under steroid therapy waiting to undergo second-stage adrenalectomy had failed. Equally doomed to failure too was the attempt to measure the "expected" zero production in the patient after adrenalectomy. But the attempt brought to light the existence of an interconvertible relationship between cortisone and hydrocortisone and as such the cause of the failure became known. It was impossible to measure the endogenous hydrocortisone production rate by the blood ^{14}C test under these circumstances. After the event it was painfully realised that the purpose of this particular investigation could have been fulfilled if the urine ^{14}C method for the measurement of production had been used. If this had been done, the pre-operative production value minus the quantity of steroid administered to the patient during the last 24 hours would have revealed the production rate of the remaining adrenal gland. And likewise, the post-operative production value /

value minus the quantity of steroid the patient had received during the last 24 hours would have revealed whether or not there existed an extra-adrenal supply of hydrocortisone. Furthermore these tests need not have to be carried out during the days immediately before or after the operation; it may be carried out at a time when the patient is receiving a constant steroid dose of 25 mg/day, a period long before and after the operation. Certainly this problem merits further investigation and the answer one gets from using the urine ^{14}C test will be not only 80 - 100% accurate but also most rewarding.

Nitrogen, Water and Electrolyte Response.

The most notable changes which follow surgical trauma have been the occurrence of oliguria, retention of sodium and loss of potassium and nitrogen. The occurrence of oliguria may be explained by a decrease in glomerular filtration due to a fall in blood flow and by an increased tubular reabsorption caused perhaps by increased liberation of pituitary antidiuretic hormone. The increased nitrogen excretion arises from a rapid catabolism of protein and the negative nitrogen balance during this period cannot be prevented, although it may be modified or minimised by suitable preparation of the patient such as starving him or paradoxically perhaps, by protein feeding in sufficient quantity. The duration and extent of the "catabolic phase" which represents the body's inherent response to trauma is related to the nutritional state of the patient and to the gravity of the wound. The potassium loss which occurs promptly after injury does not persist as long as either the increased nitrogen excretion or the reduced sodium output and although it was alleged to be a necessary accompaniment of the post-operative nitrogen excretion, it was found to occur much earlier than the "catabolic phase" and /

and the amount excreted bore no relation to the nitrogen output. It is conceivable therefore that this early potassium loss is neither due to the catabolic destruction of protein nor a response to sodium retention; that it must be of cellular origin and represents a very early part of general reaction to trauma. The most difficult to account for of the various metabolic changes following surgical trauma is that of sodium retention. It has been suggested that this retention was designed to maintain fluid tonicity due to the oliguria or to compensate for the early loss of potassium; that it was due to the ionic exchange of sodium for intracellular potassium because of increased cell permeability. But in the light of the urinary specific gravity determinations during the oliguria phase or of the time relationships between the occurrences of potassium loss and sodium retention, it is evident these proposed factors appear to play only a minor role.

Other metabolic changes.

It has also been known for some time now that the serum albumin falls abruptly after operation, so does the plasma iron concentration. Why this happens is not certain but it is unlikely that the former is due to a general protein depletion or the latter to simple bleeding or external loss. There also appears to be some disturbance of carbohydrate, fat and vitamin metabolism due to injury. Fat is rapidly oxidised probably to provide the energy necessary for the many altered metabolic processes and this contributes to a fall in body weight which is a characteristic feature after injury. It is believed that carbohydrate stores are mobilised due to the stress-induced hyperadrenalinaemia which in turn steps up the production of ACTH and consequently the secretion of adrenocortical hormones. This results in the formation of glucose /

glucose from aminoacids and inhibits carbohydrate breakdown. Associated with the "catabolic phase" there is an increased output of riboflavin and other substances derived from vitamins of the B complex. Has this been designed to provide the necessary co-enzymes for the various oxidation-reduction processes? The urinary excretion of vitamin C has also been observed to fall after injury. Is this change intended to facilitate healing of the wound since it is known that deficiency of this vitamin gives rise to poor prognosis? Whether the fall in excretion of ascorbic acid after injury is due to an increased retention by the tissue or to a greatly increased oxidative breakdown is still uncertain. What is certain is that these metabolic changes just discussed do merit further investigations.

Mechanism Responsible for the Metabolic Changes.

For some time now the mechanisms by which these metabolic changes are initiated and sustained have received considerable attention. Trauma, be it due to surgery or accident, evokes a real increase in adrenocortical secretion; this gives rise to elevated plasma steroid levels and to increased urinary steroid excretion. There is also an increased liberation of aldosterone and of antidiuretic hormone. Albright's proposal that these increased hormone secretions are solely responsible for the post-surgical metabolic changes is now unacceptable because it has been shown that patients kept on a constant supply of hormone and subjected to adrenalectomy and hypophysectomy displayed the usual metabolic changes after operation. However, as had already been mentioned the "permissive action" of the adrenal gland implies that the initiating causes of the response must be sought elsewhere and /

and it has been suggested that a closer examination of the sequence of events following cell damage - which gives rise to changes in the distribution of water and electrolytes and possibly the liberation of toxic substances thus causing further metabolic changes may reveal some satisfactory explanation for the various metabolic phenomena which follow injury, surgical or otherwise.

Summary

Summary.

1. Two methods were used for the measurement of the endogenous hydrocortisone production rate in Man. The blood method largely followed that proposed by Peterson and Wyngaarden (1956) and the urine method, to a great extent, that proposed by Cope and Black (1958). A dose of 10 - 20 μ g hydrocortisone containing 0.2 μ c of radioactivity was administered to the subject under investigation and from the measurement of the rate of dilution the administered hydrocortisone underwent with the hydrocortisone produced by the subject's own adrenals, the endogenous hydrocortisone production was calculated. All radioactivity counting was carried out using a Panax Liquid Scintillation Counter which recorded a counting efficiency of 70% and a toluene base liquid phosphor. There appeared to be good agreement in the results obtained by the two methods. But while agreeing that a dose of 0.2 μ c was quite sufficient for the measurement of production from 24 hour urine, the amount of radioactivity present in the plasma, 0 - 2½ hours after administration of the dose, was too small for accurate measurement and experience suggests that a dose of 1 - 1.5 μ c instead of 0.2 μ c will not only considerably facilitate measurement of production by the blood method but also render it more reliable.

2. After the establishment of the "normal" production rate patterns, application was made to the investigation of 13 cases of Cushing's syndrome, 4 cases of Hypoadrenalism and 7 cases of Hirsutism. From five patients with no apparent endocrine abnormality (three convalescent and two pre-surgical cases) a normal production range of 16 - 30 mg/day was obtained by the blood technique and from a series of seven healthy young adults a production of 9 - 19 mg/day was obtained by the urine technique. Eight of the /

the thirteen Cushing's cases registered an elevated hydrocortisone production and when compared with the 17-KGS and 17-KS values obtained at the same time, the production values have much to recommend the ^{14}C test as the method of choice for assessing adrenal function especially in this clinical condition where the changes are often so moderate that they may not be reflected in the 17-KGS and 17-KS excretion values. Two low and two normal production values were observed in the four hypoadrenal cases investigated but the corresponding low or normal values of 17-KGS and 17-KS excretions obtained in these cases suggest that in hypoadrenal states these routine laboratory tests are reliable enough to reflect the decreased state of adrenal activity. A normal production was observed to occur in six of the seven Hirsute cases investigated when measured by the urine method. But measurements carried out by the blood method revealed values which, although agreed well with those obtained from urine studies, surprisingly indicated a sub-normal production when judged by a normal production of 16 - 30 mg/day. In the absence of a normal production range obtained from healthy women of comparable age group it has not been possible to assert whether in fact these blood production values are normal or indeed sub-normal.

3. A real post-operative increase in hydrocortisone production was found to occur in 4 patients undergoing surgery which did not involve the removal of the adrenal glands. At the same time the usual nitrogen, water and electrolyte changes which have been observed to follow injury also occurred in these patients when balance studies were carried out before and after operation. These investigations were carried out with a view to obtaining further information regarding the role of the adrenal gland in the metabolic response to /

to surgery. An attempt to measure the pre- and post-operative hormone production in a patient undergoing a second-stage adrenalectomy was also made. The attempt failed in the fulfillment of its purpose because the constant but large dose of cortisone acetate which the patient was receiving during the immediate period before and after operation underwent an interconvertible relationship with hydrocortisone and as such it became impossible to measure the endogenous hydrocortisone production either before or after the second-stage adrenalectomy. However, a greatly diminished hydrocortisone pool was observed after the operation and in the face of a potassium and nitrogen loss with a concomitant sodium retention occurring during the same period, the role of the adrenal gland as "permissive" of these metabolic responses after surgery became once again very suggestive.

References

- Albright, F. (1942-43) Harvey Lecture 38, 123.
- Anderson, E., Haymaker, W., Joseph, M., (1938). Endocrinology, 23, 35.
- Ariel, I.M., and Miller, F., (1950). Surgery, 28, 716.
- Bell, C.G., and Hayes, F.N., (1958). Liquid Scintillation Counter.
London: Pergamon Press Ltd.
- Benedict, F.G., (1915). Publ. Carneg. Instn., No.203.
- Birke, G., Franksson, C., and Plantin, L., (1955). Acta endocr.
Kbh., 18, 201.
- Birke, G., Diczfalussy, E., Plantin, L., (1958). J. clin. Endocrin.
and Metab., 18, 736.
- Bixenkron-Moller, N., (1949). Acta chir scand., 97, 300.
- Black, D.A.K., and Milne, M.D., (1952). Lancet, 1, 244.
- Bongiovanni, A.M., Eberlein, W.R., Grumbach, M.M., Van Wyk, J.J. and
Clayton, G., (1954). Proc.Soc. exp. Biol., N.Y., 87, 282.
- Bondy, P.K., Abelson, D., Scheuer, J., Tsen, T.K.C. and Upton, V.,
(1957). J. biol Chem., 224, 47.
- Bondy, P.K., and Altrock, J.R., (1953). J. clin. Invest., 32, 703.
- Brooks, R.V., McSwiney, R.R., Prunty, F.T.G. and Wood F.I.Y., (1957).
Amer.J. Med., 23, 391.
- Brooks, R.V., Mattingly, D., Mills, I.H. and Prunty, F.T.G., (1960).
Brit. med.J., 1, 1294.
- Brooks, R.V., Prunty, F.T.G., (1960), Dec. J. Endocrin. 21, 263.
- Brown, W.O. and Bradman, H.G., (1961). Biochem. J., 78, 571.
- Bush, I.E. (1952). Biochem. J., 50, 370.
- Chen, C. and Tewell, H.E., (1951). Fed.Proc., 10, 377.
- Coller, F.A., Campbell, K.N., Vaughan, H.H., Iob, V. and Moyer, C.A.,
(1944). Ann. Surg., 119, 533.
- Coller, F.A., Rees, V.L., Campbell, K.N., Iob, V. and Moyer, C.A.,
(1943). Ann. Surg., 118, 717.
- Cooper, D.R., Iob, V. and Coller, F.A., (1949). Ibid, 129, 1.
- Cope, C.L. and Black, E.G., (1959). Brit. med. J., 2, 1117.
- Cope, C.L. and Black, E.G., (1958). Clin. Sci., 17, 147.

- Cope, C.L. and Black, E.G., (1958). Brit. med. J., 1, 1020.
- Cope, C.L. and Black, E.G., (1959). J. Obstet. Gynae. Brit. Emp. 66, 404.
- Cope, C.L. and Hurlock, B., (1954). Clin. Sci., 13, 69.
- Corcoran, A.C. and Page, I.H., (1948). J. Lab. clin. med., 33, 1326.
- Cuthbertson, D.P., (1930). Biochem. J., 24, 1244.
- Cuthbertson, D.P., (1932). Quart. J. med., 25, 233.
- Co Tui, Wright, A.M., Mulholand, J.H., Bacham, I. and Breed, E.S., (1944). Ann. Surg., 119, 815.
- Daughaday, W.H., Jaffe, H. and Williams, R.H., (1948). J. clin. Endocrin., 8, 244.
- Davidson, J.D. and Fiegelson, P., (1957). Int. J. appl. Radiat. Isotopes, 2, 1.
- de Courcy, C., Bush, I.E., Gray, C.H. and Lunnon, J.B., (1953). J. Endocrinol., 9, 401.
- Diczfalnczy, E., Plantin, L., Birke, G. and Westman, A., (1955). Acta Endocr. Kbh., 18, 356.
- Dorfman, R.I., (1950). Hormone Assay, pg. 325. Ed. C.W. Emmers, Academic Press, New York.
- Dorfman, R.I., (1954). Rec. Progr. Hormone Res., 9, 5.
- Dorfman, R.I., Shipley, R.A. and Howitt, B.M., (1943). Amer. J. Physiol., 139, 742.
- Dorfman, R.I., Howitt, B.M., Shipley, R.A. and Abbott, W.E., (1944a). Endocrinology, 35, 15.
- Dorfman, R.I., Howitt, B.M. and Shipley, R.A., (1944b). Endocrinology, 35, 49.
- Dorfman, R.I., and Shipley, R.A., (1956). The androgens, Chapman and Hall, London.
- Drekter, I.J., Pearson, S., Bartzak, E. and McGavack, T.H., (1947). J. clin. Endocrin., 7, 794.
- Drekter, I.J., Heisler, A., Scism, G.R., Stern, S., Pearson, S. and McGavack, T.H., (1952). J. clin. Endocrin. and Metab., 12, 55.
- Dudley, H.A.F., Robson, J.S., Smith, M. and Stewart, C.P., (1957). Clin. Chem. Acta., 2, 461.
- Dustan, H.P., Mason, H.L., and Corcoran, A.C., (1953). J. clin. Invest., 32, 60.

- Eik-Nes, K., Nelson, D.H., Samuels, L.T., (1953). J. clin. Endocrin. Metab., 13, 1280.
- Elman, R., Weichselbaum, T., Moncrief, J.C. and Margraf, H.W. (1955) Arch. Surg.Chicago, 71, 697.
- Elman, R., Lemner, R.A., Weichselbaum, T., Osen J.G. and Yore, R.W., (1949). Ann. Surg., 30, 703.
- Finkelstein, M., (1952). Nature, London, 169, 929.
- Forbes, A.P., Donaldson, E.C., Reifenstein, E.C. Jr. and Albright, F. (1947). J. clin. Endocrin, 7, 264.
- Franksson, C., Gemzell, C.A., von Euler, U.S., (1954). J. clin. Endocrin., 14, 608.
- Fukushima, D.K., Leeds, N.S., Bradlow, H.L., Kritchevsky, T., Stokem, M.B. and Gallagher T.F., (1955), J. biol. Chem., 212, 449.
- Glenn, E.M. and Nelson, D.H., (1953). J. clin. Endocrin. and Metab., 13, 911.
- Gold, N.I., Singleton, E., MacFarlane, D.A., Moore, F.D., (1958). J. clin. Invest., 37, 813.
- Grollman, A. and Prior, W.M., (1932-33), Proc. Soc. exp. Biol. N.Y., 30, 669.
- Grossman, C.M., Sappington T.S., Burrows, B.A., Laviates, P.H. and Peters, J.P., (1945). J.clin.Invest., 24, 523.
- Guinn, V.P. (1958). Liquid Scintillation Counting, pg. 166. Ed. C.G. Bell and F.N. Hayes, Pergamon Press, London.
- Habif, D.V., Papper, E.M., Fitzpatrick, H.F., Lawrence, R., Symthe C. and Bradley, S.E., (1951). Surgery, 30, 241.
- Hardy, J.D. and Ravdin, I.S., (1952). Ann. Surg., 135, 345.
- Hardy, J.D., Turner, M.D. and Jackson, (1957). Surgery, 42, 194.
- Hardy J.D. and Turner, M.D., (1956). Surg. Forum, 7, 139.
- Hartman, F.A., MacArthur, C.G. and Hartman, W.E., (1927). Proc.Soc. exp. Biol, 25, 69.
- Heard, R.D.H. and Sobel, H., (1946). J. biol. Chem., 165, 687.
- Hellman, L., Bradlow, H.L., Adesman, J., Fukushima, D.K., Kulp, J.L. and Gallagher, T.F., (1954). J.clin.Invest., 33, 11.

- Helmreich, M.L., Jenkins, D. and Swan, H., (1957). Surgery, 41, 895.
- Holland, B.C. and Stead, E.A., (1951). Arch. intern. Med., 88, 571.
- Howard, J.E., Bigham, R.S. Jr., Eisenberg, H., Wagner, D. and Bailey
(1946). John Hopkins Hosp. Bull., 78, 282.
- Howard, J.E., Parson, W., Eisenberg, H.S., Stein, K.E. and Reidt,
(1944). John Hopkins Hosp. Bull., 75, 156.
- Hume, D.M. and Nelson, D.H., (1954). Surg. Forum, 5, 568.
- Ingle, D.J., Ward, E.O. and Kuizenga, M.H. (1947). Amer. J. Physiol.
149, 510.
- Ingle, D.J., (1951). Amer. intern. Med., 35, 652.
- Kallman, H. and Furst, M., (1950). Phys. Rev. 79, 857.
- Knowlton, A.I., (1952). Med. Clin. N.Amer., 36, 721.
- Le Quesne, L.P. and Lewis, A.A.G., (1953). Lancet 1, 153.
- Levell, M.J., Mitchell, F.L., Paine, C.G. and Jordan, A., (1957),
J. clin. Path., 10, 72.
- Levenson, S.M., Davidson, C.S., Lund, C.C. and Taylor, F.H.L., (1945)
Surg. Gynae. Obstet., 80, 449.
- Liddle, G.W., Estep, H.L., Kendall, J.W., Williams, W.C. and Townes,
A.W., (1959). J. clin. Endocrin. Metab., 19, 8.
- Limbert, E.M., Power, M.H., Pemberton, J. de J. and Wakefield, E.G.
(1945). Ibid, 80, 609.
- Lindsay, A.E., Migeon, C.J., Nugent, C.A., and Brown, H., (1956).
Amer. J. Med., 20, 15.
- Llaurado, J.G. and Woodruff, M.F.A., (1957). Surgery, 42, 313.
- Long, C.N.H., Katzin, B., Fry, E.G. (1940). Endocrinology, 26, 309.
- Lowenstein, B.E., Cocoran, A.C. and Page I.H., (1946). Endocrinology
39, 82.
- MacPhee, I.W., (1953). Brit. med. J. 1, 1023.
- Manery, I. and Solandt, (1943). Amer. J. Physiol., 138, 499.
- Markley, K., Bocanegra, M., Ego-Aguirre, E., Chiappori, M. and Moral
G., (1960). Surgery, 47, 389.
- Marks, L.J., Donovan, M.J., Duncan, F.J., Karger, R., (1959), J. cli.
Endocrinol, 19, 1458.

- Mason, H.L. and Engstrom, W.W., (1950). *Physiol. Rev.*, 30, 321.
- Migeon, C.J., Sandberg, A.A., Decker, H.A., Smith, D.F., Paul, A. Carl.,
Samuels, L.T., (1956). *J. clin. Endocrin. Metab.*, 16, 1137.
- Mittelman, A. and Barker, H.G., (1956). *Surg. Forum*, 7, 133.
- Moore, F.D. and Ball, M.R. (1952). *The Metabolic response to Surgery*,
Thomas, Springfield, Ill.
- Morris, C.J.O.R. and Williams, D.C. (1953). *Biochem. J.*, 54, 470.
- Moxham, A. and Nabarro, J.D.N., (1956). *J. clin. Path.*, 9, 351.
- Moyer, C.A., (1950). *Surgery*, 27, 198.
- Nabarro, J.D.N., Moxham, A. and Walker, G., (1958). *J. clin. Endocrin. Metab.*, 18, 586.
- Neher, R., (1958). *Adv. Clin. Chemistry Vol.I*, pg.128, Ed. H. Sobotka
and C.P. Stewart, Academic Press Inc. N.Y.
- Neher, R., Wettstein, A., (1951). *Helv. chem. Acta*, 34, 2278.
- Nelson, D.H. and Samuels, L.T., (1952). *J. Clin. Endocrin. Metab.*,
12, 519.
- Nelson, D.H. and Harding, B., (1952). *Fed. Proc.* 11, 379.
- Norymberski, J.K., (1952). *Nature, Lond.*, 170, 1074.
- Nowaczynski, W.J. and Koiw, E. (1957). *J. Lab. Clin. Med.*, 47, 815.
- Osman, L., (1961). Ph.D. Thesis, University of Edinburgh.
- Patterson, J., (1960). *Adrenal Cortex*, pg. 74, Ed. G.K. McGowan and
M. Sandler, Pitman Med. Pub.Co.Ltd., London.
- Perla, D. and Marmosten-Gottesman, J., (1931). *Proc.Soc.exp. Biol.N.Y.*,
28, 1024.
- Perry, W.F. and Gemmell, J.B., (1949). *Canad. J.Res.* 27, 320.
- Peters, J.P., (1944). *Fed. Proc.*, 3, 197.
- Peterson, R.E. and Wyngaarden, J.B., (1956). *J.clin.Invest.*, 35, 552.
- Peterson, R.E., Karrer, A. and Guerra, S.L. (1957). *Analyt.Chem.*,
29, 144.
- Peterson, R.E., Wyngaarden, J.B., Guerra, S.L., Brodic, B.B., and
Bunim, J., (1955). *J. clin.Invest.*, 34, 1779.
- Peterson, R.E., (1959). *Rec. Prog. Hormone Res.*, 15, 231.

- Schilling, J. A., McCoord, A.B., Clansen, S.W., Whelan, T.J. and Kuykendall, S.J., (1953). *Surg.Gynec.Obstet.*, 97, 162.
- Selye, H. and Schenker, V., (1938), *Proc. Soc. exp. Biol. N.Y.*, 39, 518.
- Shipley, R.A., Dorfman, R.I., Buchwald, E. and Ross, E., (1946). *J. Clin. Invest.*, 25, 673.
- Silber, R.H. and Porter, C.C., (1954). *J. biol. Chem*, 210, 923.
- Silber, R.H., (1955). *Clin. Chem.*, 1, 234.
- Simpson, S.A. and Tait, J.F., (1952). *Endocrinology*, 50, 150.
- Speirs, R.S., and Meyer, R.K., (1951). *Endocrinology*, 48, 316.
- Steenberg, R.W., Lennihan, R. and Moore, F.D., (1956). *Ann. Surg.*, 143, 180.
- Steenberg, R.W., Smith, L.L. and Moore, F.D., (1961). *J. clin. Endocrin. Metab.*, 21, 39.
- Stewart, G.M. and Rogoff, J.M., (1927). *Science*, 66, 327.
- Stewart, C.P., (1955). *Bull.Soc.Inter.Chir.* 14. Report of XVI Congress International de Chirurgie, Copenhagen.
- Stewart, C.P., Dudley, H.A., Smith, M., Robson, J.S., (1957) *Scand. J.Clin.Lab.Invest.* Vol.10, Supplement 31.
- Stewart, C.P., (1961). *Proceedings of Ass.Clin.Biochemists*, Vol.1, No.4, pg. 78.
- Stitch, S.R., (1959), *Biochem.J.*, 73, 287.
- Sulkowitch, H., Rutenberg, A.M., Lesses, M.F., Garjill, S. and Seligman, A.M., (1955). *New Engl.J.Med.*, 252, 1070.
- Swan, H., Jenkins, D., Helmreich, M.L., (1957). *Surgery*, 42, 202.
- Sweat, M.L., (1954). *Analyst.*, 26, 773.
- Sweat, M.L., (1955). *J. clin. Endocrin. Metab.*, 15, 1043.
- Talbot, N.B., Saltzman, A.H., Wixom, R.L. and Wolfe, J.K., (1945). *J. biol. Chem.* 160, 535.
- Thorn, G.W., Jenkins, D., Laidlaw, J.C., Gock, F.C. and Reddy, W., (1953). *Trans.A.Amer.Physic*, 66, 1953.
- Tomiza, H.H., Nerchara, H.T., Gibbons, C.A. and Williams, R.H., (1954). *Proc.Soc.exp. Biol. N.Y.*, 85, 51.
- Tompsett, S.L. and Smith, D.C., (1954). *J.clin.Endocrinol.*, 14, 922.

- Tyler, F.W., Schmidt, C.P., Eik-Nes, K., Brown, H. and Samuels, L.T.,
(1954). J.clin.invest., 33, 1509.
- Venning, E.H., Kazmin, V.E. and Bell, J.C., (1946), Endocrinology,
38, 79.
- Venning, E.H., Hoffman, M.M. and Browne, J.S.L., (1943). J. biol.
Chem., 148, 455.
- Venning, E.H., Hoffman, M.M. and Browne J.S.L. (1944). Endocrinology,
35, 49.
- Venning, E.H., Browne, J.S.L. (1947). J.clin.Endocrinol., 7, 79.
- Vestergaard, P., (1951). Acta Endocrin. Kbh., 8, 193.
- Virtue, R.W., Helmreich, M.L. and Gainza, E., (1957). Surgery, 41, 549.
- Weil, P. and Browne, J.S.L., (1939). Science, 90, 445.
- Weil, P. and Browne, J.S.L., (1940). J. clin. Invest., 19, 772.
- Werner, S.C., (1948). J.clin. Invest., 27, 561.
- Wieland, H., Straub, W. and Dorfmueller, T., (1929). Hoppe-Seyl, Z.,
186, 97.
- Wilkinson, A.W., Billing, B.H., Nagy, C. and Stewart C.P., (1950).
Lancet, 1, 533.
- Wilkinson, A.W., Billing, B.H., Nagy, C. and Stewart, C.P., (1950),
Lancet, 11, 135.
- Wilkinson, A.W., Billing, B.H., Nagy, C. and Stewart, C.P., (1949).
Lancet, 1, 640.
- Windfield, J.M., Fox, C.L. and Mersheimer, W.L., (1951). Ann. Surg.,
134, 626.
- Wintersteiner, O. and Pfiffner, J.J., (1936). J. biol. Chem., 116, 291.
- Zaffaroni, A., (1953). Rec. Progr. Hormone Res., 8, 51.